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Sinusoidal Dilatation Occurring in Livers of Mice with a Transplanted Testicular Tumor.* (18007)

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Growth of certain transplanted ovarian tumors in mice is associated with an increased blood volume and sinusoidal dilatation in the liver, spleen, and adrenal glands(1,2,3). This phenomenon is not related either to the hormonal activity of the tumor or to an associated viral infection(4). The changes that are seen occur consistently with certain granulosa-cell tumors that have been induced in this laboratory by the homologous transplantation of an ovary into the spleen(5).

Experimental. Hybrid mice (A x C_3H) that carried subcutaneous or intraperitoneal transplants of a testicular interstitial cell tumor ($3AC_1SS$)(6) that arose in a stilbestrol-treated hybrid mouse of similar parentage were used. The tumor had been transplanted for 20 "transfer" generations before the present observations began. Originally the tumor grew only in estrogen-treated mice but subsequently grows in both male and female mice. Although sinusoidal changes similar to those occurring in mice bearing granulosa cell tumors may have occurred prior to the twentieth "transfer" generation, adequate studies were not made.

Animals bearing subcutaneous transplants of the testicular tumor did not show as extensive dilatation of the hepatic sinusoids as was observed in animals bearing intramesenteric transplants of comparable size.

The livers of these animals developing sinusoidal dilatation were congested and heavier than those of the controls; their surfaces were mottled and bled easily when touched. Microscopically, the hepatic sinusoidal dilation ranged from slight to extreme dilatation with sinusoids isolating individual cords of liver cells. The dilatation began in the peri-lobular portal areas and extended toward the central veins which remained intact until gradually the entire architecture of the liver was destroyed. There was no increase in the number of mitoses among the liver cells and there was no polymorphonuclear or round cell infiltration. There was a slight increase in the number of Kupfer cells; the small bile ducts were not dilated. The spleens of these animals were

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† Post-doctorate Research Fellow, National Institute of Health.

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TABLE I.
Organ Weights and Degree of Sinusoidal Dilatation in Mice Bearing a Transplanted Interstitial-Cell Testicular Tumor.

Animal No.	Liver wt, g	Spleen wt, g	Tumor wt, g	Sinusoidal dilatation* Liver	Sinusoidal dilatation* Adrenal
		Subcutaneous tumors			
T_{22}-1 ♂	2.47	.26	2.24	++	0
T_{22}-2 ♂	2.12	.29	6.44	+++	0
T_{22}-3 ♂	2.17	.40	6.80	+++	0
18 ♀	2.39	.47	6.98	+	0
32 ♀	2.87	.77	7.58	+	0
D-1 ♂	2.87	.46	7.60	++	0
1 ♂	3.65	.54	8.80	+++	++
2 ♀	2.72	.75	10.40	+	0
Range	2.12-3.65	.26-.77	2.24-10.40		
(8) Avg wt	2.66	.49	7.11		
		Intra-mesenteric tumors			
6 ♀	2.86	.52	3.71	+	0
3 ♂	1.74	.34	3.95	+	0
7 ♀	2.12	.37	5.27	+	++
4 ♀	2.40	.52	5.30	++	+
1 ♂	2.69	.41	5.66	+++	0
12 ♀	2.31	.27	5.70	++	0
15 ♂	2.06	.27	5.90	++++	++++
6 ♀	2.21	.44	6.67	+++	0
14 ♀	2.08	.30	6.79	++	0
11 ♂	2.32	.37	7.96	++++	+++
2 ♂	2.45	.46	8.92	+++	+
8 ♂	1.80	.56	9.17	+++	0
4 ♂	2.14	.67	9.88	+++	++++
16 ♂	2.00	.27	10.93	++++	++++
Range	1.74-2.86	.27-.67	3.71-10.93		
(14) Avg wt	2.23	.41	6.84		
	Controls				
1 ♀	1.48	.12			
2 ♀	1.81	.20			
3 ♂	1.57	.18			
4 ♂	1.70	.17			
5 ♂	1.65	.19			
6 ♂	1.50	.16			
7 ♂	1.41	.20			
Range	1.41-1.81	.12-.20			
(7) Avg wt	1.58	.17			

* 0 to + Beginning sinusoidal dilatation.
+ to ++ Early sinusoidal dilatation.
++ to +++ Moderate sinusoidal dilatation.
+++ to ++++ Extreme sinusoidal dilatation.

likewise enlarged and congested. Microscopically, they showed marked congestion of the red pulp and no increase in hematopoiesis.

The changes in the adrenal glands were most striking. Grossly, the adrenal glands were enlarged several times and had a homogenous red-purple color. Histologically, the sinusoids at the cortico-medullary junction were dilated and distended into the cortex, disrupting the normal architecture of the cortex until only scattered individual cortical cells remained. The cells of the medulla remained essentially intact.

Castrate animals did not reveal any hormonal activity of the tumor. There was no correlation between the sinusoidal dilatation and the amount of necrosis observed in the tumor; neither was there any correlation with the rate of growth nor with the sex of the animal. The most extensive sinusoidal

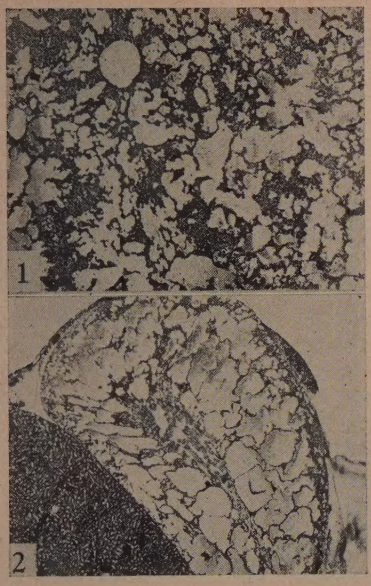

FIG. 1.

Section of liver of tumor bearing animal No. 15 ♂ AC_1. showing ++++ sinusoidal dilatation. Mag. 32 mm objective and 8x ocular.

FIG. 2.

Section of adrenal gland of tumor bearing animal No. 15 ♂ AC_1, showing ++++ sinusoidal dilation. A small section of the kidney is included in the lower left corner. Mag. 32 mm objective and 8 x ocular.

dilatation occurred in mice bearing intramesenteric transplants exceeding 5 g at about 4-6 weeks after transplantation.

Discussion. The pathogenesis of these changes remains to be described. Their occurrence with tumors that have no apparent hormonal activity tends to eliminate the possibility of the steroid hormones as the causative agent. They may be due to a hormonal imbalance as a hepatic perilobular sinusoidal dilatation has been described as occurring in the livers of hyperthyroid rats(7); however, histological examination of the thyroids of the animals in this experiment revealed normal histology. This tumor may be producing a specific protein factor or inducing a deficiency state yet to be recognized.

Summary. 1. Mice having a transplanted interstitial cell testicular tumor developed sinusoidal dilatation in the liver, spleen and adrenal glands. 2. These changes were more marked in animals with intra-mesenteric transplants than in animals with subcutaneous transplants. 3. This testicular tumor did not have any hormonal activity in castrate animals.

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Development of Hypertension in the Adrenalectomized Nephritic Rat Maintained on NaCl.* (18008)

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From reports in the literature, it is difficult to determine whether adrenal cortical hormones are essential for the development or maintenance of experimental renal hypertension.

In the dog, Goldblatt(1) has stated that "bilateral adrenalectomy eliminates completely the response to constriction of the renal arteries." Page's(2) and Blalock's observations(3) are in accord with this statement. On the other hand, Rogoff *et al.*

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1. Goldblatt, H., *Ann. Int. Med.*, 1937, vII, 69.

2. Page, I. H., *Am. J. Physiol.*, 1938, v122, 352.

3. Blalock, A., and Levy, S. E., *Ann. Surg.*, 1937, v106, 826.

4. Rogoff, J. M., Nixon, E. N., and Stewart, G. N., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 57.

TABLE I.
Allocation of Animals.

Group	% NaCl in diet	% NaCl in drinking water	Operation	Rabbit anti-rat kidney serum	No. of rats	
					Allocated	Incl. in data
1	1.0	0	0	0	10	10
2	1.7	.9	0	0	10	10
3	1.7	.9	Bilateral adrenalectomy	0	22	20*
4	1.0	0	0	.8 cc	10	10
5	1.7	.9	Bilateral sham adrenalectomy (9 rats) None (26 rats)	.8 or .9 cc	35	34†
6	1.7	.9	Bilateral adrenalectomy	.7, .8, or .9 cc	58	21‡

* One rat died during operation.
1 rat omitted because of gross adrenal tissue.
† 1 rat omitted from data on blood pressure due to gangrene of tail tip.
‡ See Table II.

(4), have reported that hypertension does develop in the dog following the combined operation of clamping the renal arteries and bilateral adrenalectomy, and persists to death of the animal in adrenal insufficiency. Collins and Wood(5) agree with Rogoff that constriction of the renal arteries of adrenalectomized dogs results in hypertension, but they noted further that adrenalectomy in dogs with previously established hypertension results in a gradual reduction in blood pressure. In the rat Gaudino(6) reported that bilateral adrenalectomy usually resulted in a fall in the blood pressure of previously hypertensive animals, and in the few animals in which hypertension reappeared he has been able to demonstrate residual adrenal tissue in all but one.

The present studies were undertaken to determine whether bilateral adrenalectomy alters the blood pressure response of the rat to the type of renal damage induced by cytotoxic serum. The data comprise the accumulated findings from 3 separate series of experiments carried out over the past 15 months.

Methods. 145 rats of the Long-Evans strain were employed, all but 13 of which were males. The 13 females were divided evenly between Groups 3, 5 and 6. The ages of all rats ranged from 53-76 days at the beginning of the experimental period. The allocation of the animals is presented in Table I. Group 1 served as a control and received the laboratory stock diet(7); Group 2 received a modification of this diet to insure a high NaCl intake, as did Group 3, which in addition were bilaterally adrenalectomized. Of the remaining Groups 4, 5 and 6, all received rabbit anti-rat kidney serum, Group 4 being maintained on stock diet and Groups 5 and 6 on this diet modified to contain a high salt content. In addition, bilateral sham adrenalectomy was performed in 9 of the 35 rats of Group 5, while all the rats of Group 6 were bilaterally adrenalectomized.

Bilateral adrenalectomy was performed on the rats of Groups 3 and 6 at the start of the experiments by one of the authors (H.C.S.), under ether anesthesia without aseptic precautions. The adrenals were removed with forceps through bilateral lumbar incisions, care being taken to remove the entire periadrenal fat intact without manipulating the gland itself. The operation was followed im-

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mediately by a clysis of 5 cc of normal saline and the animals were then maintained on a high NaCl intake as indicated in Table I. Sham adrenalectomy consisted of similar bilateral lumbar incisions through which the periadrenal fat was manipulated with forceps but not removed.

Blood pressure determinations were made on all animals prior to the beginning of the experiment and thereafter at intervals of one to 2 weeks. Two methods of determining blood pressure were employed: (1) the indirect plethysmographic method described by Williams, Harrison and Grollman(8) and modified by Sobin(9), in the first 2 series of experiments; and (2) the indirect microphonic manometer method described by Friedman and Freed(10), in the third experiment. Since preliminary determinations made with both methods on a series of 19 rats having blood pressures ranging from 100-212 mm/Hg showed no greater discrepancy than the limitation of error in either individual method, the results with the two methods have been considered comparable and reported together. With both methods, each individual blood pressure reading recorded is the average of ten successive determinations having no greater range than 20 mm/Hg.

Renal damage was induced by the injection of rabbit anti-rat kidney serum. Two different pools of sera, of comparable potency, were employed. The injections were given intravenously in divided doses over a 3-day period. In the adrenalectomized rats of Group 6, injections were begun 18 days post-operatively; in the intact Group 5, injections were begun 18 days after the institution of the high NaCl intake; and in the intact Group 4, at a comparable date.

During the experimental period, the animals were weighed at frequent intervals. The serious difficulties encountered in working with adrenalectomized animals are apparent in Table II. Quantitative urinary albumin was determined(11) on all but 3 animals on one or more occasions. At the end of the experimental period, *i.e.*, after 7 to 9 weeks, the animals in the unoperated Groups 1, 2 and 4, as well as 25 rats from Group 5 were sacrificed. The remaining 10 rats of Group 5 were subjected to a 21-day period of NaCl withdrawal before sacrifice. In the control adrenalectomized Group 3, 4 died during the

TABLE II.
Fate of 58 Rats Allocated to Group 6.

Included in final data:	21 rats
Discarded, due to:	
Death from anesthesia during adrenalectomy	6
Death following adrenalectomy and prior to administration of serum	9
Death within 3 wks after serum administration	16
Presence of adrenal tissue	6

TABLE III.

Group	Regimen	No. of rats	Body wt, g	Kidney wt, g	Urine albumin, g/l	Instance of renal lesions
1	—	10	251	1.87	2.5	—
2	NaCl	10	246	2.03	2.5	—
3	ADX + NaCl	20	191	1.70	1.6	None of 8 examined
4	AKS	10	263	2.08	29.0	8 of 10 "
5	NaCl + AKS	35	267	2.49	20.2	33 of 33 "
6	ADX + NaCl + AKS	15* 6†	186* 252†	1.91* 2.44†	18.3* 18.2†	6 of 8 " ‡

* Non-hypertensives.
† Hypertensives.
‡ In remaining 13 animals in this group which died (spontaneously or on salt withdrawal) autopsies were performed at varying intervals after death and in these autolysis prevented distinction of mild renal damage. In the 9 of these 13 studied histologically no advanced renal lesions were seen.

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10. Friedman, M., and Freed, S. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 670.
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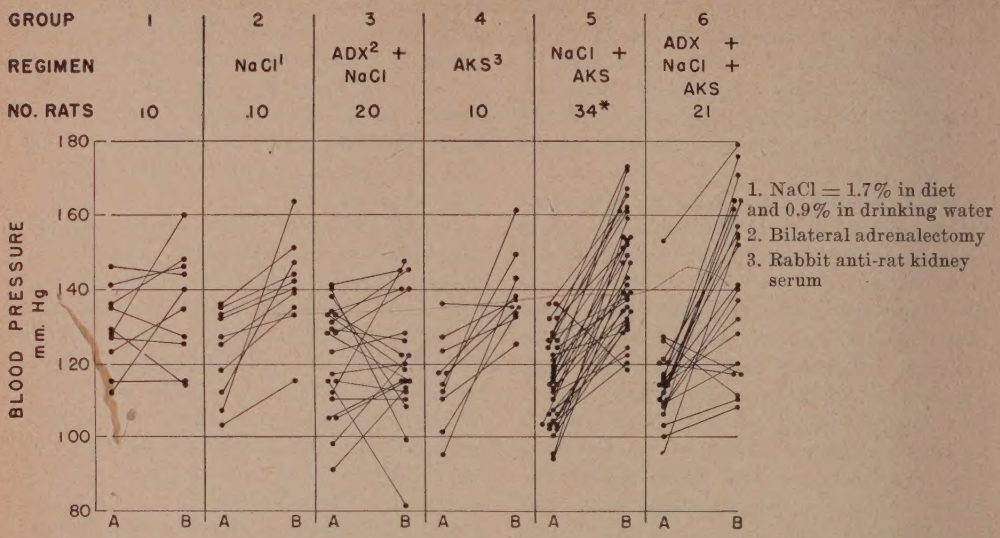

A. Preliminary readings on individual rats.
B. Maximal reading on individual rats after institution of experimental regimen
** 9 of these rats bilaterally sham adrenalectomized*

FIG. 1.
Control and maximal blood pressure readings.

period of observation, 8 were sacrificed and the remaining 8 were subjected to NaCl withdrawal. In the nephritic adrenalectomized Group 6, 5 rats died, 8 were sacrificed and the remaining 8 animals were subjected to NaCl withdrawal. At death or sacrifice, the animals were weighed and heart and kidney weights recorded. (In the adrenalectomized rats in which sodium was restricted terminally, the weight prior to the institution of this regimen has been recorded as the final body weight).

The criterion of the completeness of adrenalectomy was the absence of macroscopic or microscopic evidence of adrenal tissue at sacrifice or death. In the rats subjected to sodium withdrawal, physiological evidence of absence of the adrenals, *i.e.* the inability to survive more than 15 days without added salt was an additional criterion. One rat in Group 3 did not fulfill this last criterion, but is included because at sacrifice after 15 days of NaCl withdrawal, its serum sodium was 112 mEq/l and no adrenal tissue was found.

Results. The rats in the intact groups gained weight and remained well during the period of observation. In the adrenalectomized Groups 3 and 6, 16 animals died during the operative procedure or shortly thereafter. The average growth of the adrenalectomized animals was less than that of the intact groups although individual animals grew satisfactorily (compare final body weights of Group 3 and non-hypertensive animals of Group 6 with intact groups, Table III). In addition, 16 of the Group 6 animals died too early in the experimental period after serum administration to allow for an adequate number of blood pressure determinations (Table II). Hence the following results and the subsequent discussion concerns the 21 rats described in Table II as included in final data.

Blood pressure. Fig. 1 presents both the initial blood pressure readings on individual rats in each group and the corresponding individual maximal blood pressure readings obtained thereafter during the course of the experiment. No significant change can be seen in Groups 1 or 2 and the lowering of the blood pressure in animals of Group 3 is of

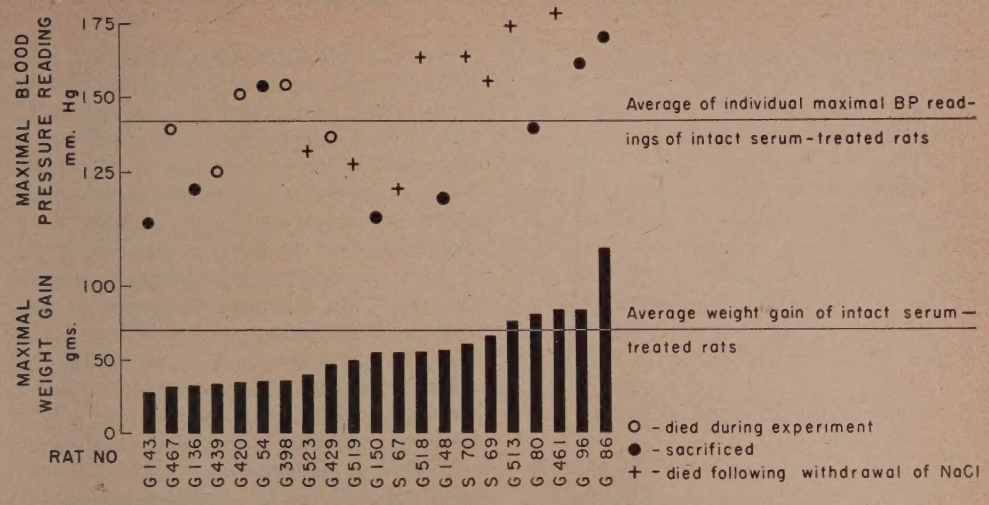

FIG. 2.
Chart of individual adrenalectomized nephritic rats in Group 6 showing maximal blood pressures and maximal weight gains during experimental period.

questionable significance. In the nephritic Group 4 on stock diet the figures are comparable to Groups 1 and 2, only one animal developed a blood pressure greater than 160 mm/Hg. Seven of the 34 nephritic rats on a high NaCl regimen, Group 5, had peak readings above 160, while 6 of the 21 similarly treated adrenalectomized rats, Group 6, had an equal degree of elevation.

In Fig. 2, the maximal blood pressures recorded for the individual adrenalectomized nephritic rats of Group 6 are plotted to show their relation to individual maximal weight gain. In addition, the average maximal blood pressure and the average maximal weight gain of the intact nephritic rats of Group 5 are indicated. From this chart, it will be seen that the highest blood pressures occurred in those rats which grew satisfactorily. The mode of death of the individual rats is likewise shown in this chart. Rats which died during the experimental period (depicted as open circles) tended, as might be expected, to be those which gained less than the average. The closed circles indicate those animals which were sacrificed, while the crosses denote those rats which were subjected to NaCl withdrawal and died following this. It will be seen that these latter animals included a number of the most hypertensive.

One rat (No. S 70) from the adrenalectomized nephritic Group 6 has been selected to show in detail the relation of satisfactory growth to the development of hypertension (Fig. 3). While this animal was gaining adequately, a progressive hypertension developed. During the ninth experimental week, the rat lost weight and following this, a significant fall in blood pressure occurred. Subsequently, following sodium withdrawal, a further 65 g weight loss ensued in the seven days prior to death.

NaCl withdrawal. The 8 adrenalectomized nephritic rats of Group 6 lost an average of 47 g (range 30-65 g) and died in 3-15 days when placed on a NaCl free diet and given tap water in place of saline to drink. The 10 intact nephritic rats of Group 5 subjected to a similar regimen of NaCl restriction, gained an average of 32 g (range 10-59 g) over 21 days and none died.

Urinary albumin. In Table III, the urinary albumin excretion in all 6 groups is shown. In all groups given the rabbit anti-rat kidney serum(4,5,6), significant albuminuria developed, indicating the presence of some degree of renal damage in these rats. *Body and kidney weights* at sacrifice also are shown in

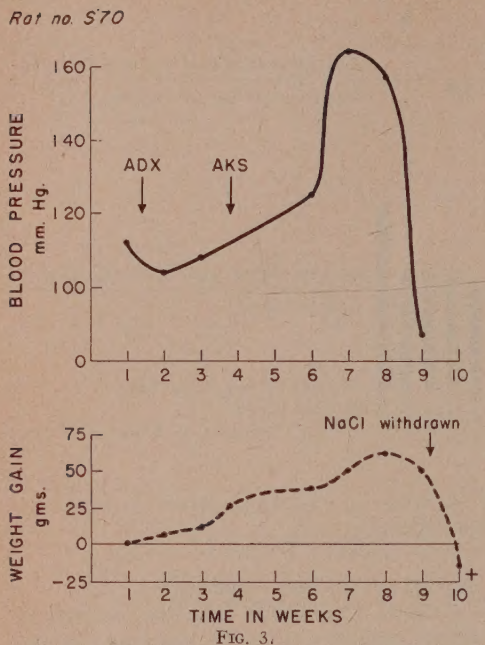

FIG. 3.

Chart of Rat No. S70—blood pressure readings and weights during experimental period.
ADX = Bilateral adrenalectomy
AKS = Rabbit anti-rat kidney serum.

Table III. From this it may be seen that the body weights of the adrenalectomized animals averaged less than that of the intact groups with the exception of the hypertensive adrenalectomized animals of Group 6. The combination of high NaCl intake and anti-kidney serum resulted in renal enlargement in the intact rats of Group 5. A similar degree of renal enlargement occurred among the hypertensive animals of Group 6. The non-hypertensive animals in this group had smaller kidneys, but since their body weights are so much less than that of Group 5, it is difficult to interpret these findings. Also included in this table is the number of rats showing histological evidence of renal damage. Renal lesions were, if anything, less frequent in the adrenalectomized rats of Group 6 than in the similarly treated intact Group 5 animals.

Summary. In response to cytotoxic serum nephritis, bilaterally adrenalectomized rats develop hypertension as frequently as do animals with intact adrenals. In order for hypertension to appear among such adrenalectomized rats, it is necessary that a satisfactory state of nutrition be maintained over a period of weeks. In our laboratory, the difficulties encountered in fulfilling this provision have been considerable in spite of the employment of a high NaCl regimen. This experience has led us to wonder whether the conflicting results in the literature relating to the question of hypertension in adrenalectomized animals may not be due, in part, to similar difficulties.

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Conjugated Linoleic Acid in Rat Tissue Lipids after Ingestion as Free Acid and as Triglyceride.*† (18009)

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The present study was conducted to test the use of conjugated linoleic acid for proposed investigations of the metabolism of glycerides by the use of carbon 14 labeled glycerol. The progression of the conjugated acid through the various tissues at successive times after its ingestion is intended as a guide for later studies with the labeled glycerol esterified with tagged acids. Advantage was taken of the experiment to test the observations of Frazer that fatty acids after ingestion go to the liver but that unhydro-

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lyzed glycerides go to the fat depots(1). The use of conjugated fatty acids as an indicator of the pathway of absorption and deposition of fat is not new. Miller and Burr(2) fed eleostearin to rats and determined its concentration in tissues at succeeding intervals. Their results were inconclusive because a new diene acid was apparently formed from the triene and the two acids were found in different concentrations. Later(3) the same laboratory reported that 5 minutes after feeding methyl or glycerol esters of conjugated linoleic acid the tagged acid constituted 26.7% of the total mucosa fatty acids. The same authors also reported(4) that after ingestion the conjugated fatty acids of corn oil were found in the acetone soluble fraction of the mucosa lipids rather than in the insoluble fraction.

Preparation of conjugated linoleic acid and its triglyceride. The fatty acids of cottonseed oil (Wesson Oil) were subjected to fractionation by the urea method(5). The diene fraction was then isomerized by the method of Bradley and Richardson(6) and the washed and dried conjugated acid esterified with glycerol by the use of the Twichell reagent at high vacuum and a temperature of 135°C. The contents of conjugated acid in the preparation were determined according to Brice *et al.*(7).

Experimental procedure. The test meals of either the conjugated acid or the triglyceride were prepared by emulsifying the preparation with tween and water so that 5 ml contained 500 mg of the conjugated acid. The material was fed by stomach tube without previous fasting at a level of 500 mg of conjugated acid per 250 g of body weight. Each experiment was performed with 2 rats in order to obtain adequate quantities of tissues for analyses. At 10, 16, 24 and 48 hours afterwards the animals were sacrificed and the blood, liver, pooled heart, lungs and kidneys and the mesenteric, perirenal and subcutaneous depot fats analyzed for their content of conjugated linoleic acid. The 10 and 16 hour experiments were performed twice; the figures in the tables being the average values.

All the tissues except blood were extracted in a Waring blendor with 10 volumes of chloroform. The mixtures were centrifuged and the partially cleared chloroform solution filtered. The chloroform was removed by vacuum distillation and approximately 100 mg samples of the lipids accurately weighed, dissolved in 100 ml of iso-octane and the density at 233 mμ determined in the Beckman spectrophotometer. Untreated animals were used as controls, the density used for final evaluation being the differences between the control and experimental tissues. Blood was analyzed by precipitation in 5 volumes of 3:1 alcohol-ether mixture. Fifteen to 20 ml of blood were obtained from a pair of rats. The solution was filtered through a small sintered glass funnel and the coagulum washed 3 times with ethyl ether. The combined extract and the washing were evaporated to dryness with reduced pressure. The impure lipids were dissolved in iso-octane. Some anhydrous Na_2SO_4 was added to assure dryness. The dry solution was filtered and made up to 100 ml. Fifty or 75 ml of the solution were used for the determination of its fat content and the spectral density determined with the remainder.

Findings. The analytical results are presented in Tables I and II. The results indicate that there is a difference in the rate and possibly in the extent of absorption, but that the pathways of absorption are probably the same. Thus, after glyceride ingestion, the maximum level of conjugated acid appears in the liver, blood and pooled organs at 16 hours while after free acid ingestion the maximum

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TABLE I.
Percentages of Conjugated Linoleic Acid in the Tissue Lipids of Rats Fed Conjugated Linoleic Acid or Its Triglyceride.

Tissue		Hrs after ingestion 10	16	24	48
		%	%	%	%
Liver	After Fatty Acid	2.02	2.75	3.05	.88
	" Glyceride	3.80	5.60	3.35	.81
Blood	After Fatty Acid	1.69	1.53	2.78	.00
	" Glyceride	2.00	2.58	1.38	.00
Organs	After Fatty Acid	0.27	0.69	0.78	.27
	" Glyceride	1.44	1.28	1.12	.65
Depot Fat	After Fatty Acid	0.12	0.13	0.28	.30
	" Glyceride	0.18	0.25	0.45	.30

TABLE II.
Percentages of Conjugated Linoleic Acid in the Depot Fats of Rats Fed Conjugated Linoleic Acid or Its Triglyceride.

Depot Fat		Hrs after ingestion 10	16	24	48
		%	%	%	%
Mesentery	After Fatty Acid	.19	.14	.39	.48
	" Glyceride	.22	.36	.52	.32
Perirenal	After Fatty Acid	.08	.18	.27	.22
	" Glyceride	.17	.22	.47	.32
Subcutaneous	After Fatty Acid	.09	.08	.17	.19
	" Glyceride	.15	.17	.36	.27

occurs at 24 hours. The maximum levels are also higher after glyceride ingestion, which would also indicate more rapid absorption. That glycerides may be absorbed more completely than fatty acids has been reported previously. Thus Lyman found that 95 to 96% of glycerol palmitate but only 80% of palmitic acid was absorbed by rats(8). The comparatively poor absorption of free fatty acid has also been reported by Perretti(9).

In Table II is given the percentage of conjugated linoleic acid in various depot fats. The average of these figures is given in Table I but the individual results are given here to show that some variations exist between the 3 depots. Labeled fat appeared more rapidly and to a greater extent in the mesentery fat, then in the perirenal and finally in the subcutaneous. It may be seen that the labeled acid increased in the depot fat for 24 hours and then leveled off, remaining at about the same concentration after 48 hours. The evidence presented here does not support the theory that fatty acids are absorbed by way of the portal blood to the liver and glycerides by way of the lymph directly to the fat depots. The higher content of conjugated acid in the fat depots after glyceride feeding is matched by its higher content in the liver and pooled organs. This shows quite clearly that the liver plays a major role in the absorption of fat as well as fatty acids. It is quite possible that many of Frazer's results on the differences in behavior of fatty acids and fats during absorption(4) can be explained on the basis of their different degrees and rates of absorption.

This slow and possibly incomplete absorption of free fatty acids still remains to

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be explained. If complete hydrolysis of fats precede absorption it should be expected that fatty acids and glycerides should be absorbed equally well, or that free acid should have an advantage. Since it has been known since the time of Frank's work that ingested fatty acids appear in the lymph as glycerides one is tempted to conclude that it is this synthesis that delays their absorption but that triglycerides need undergo no change and are absorbed as such.

However, there is an alternative interpretation. Thus the important physical state of comparatively large quantities of free fatty acid in the lumen of the intestine may be pronouncedly different from that of fat plus a small amount of fatty acid slowly released by hydrolysis and constantly removed by absorption. The resynthesis of fat from fatty acid is an uncontested fact. The requisite glycerol for this resynthesis is on hand from a recently hydrolyzed fat, but must first be synthesized in or carried to the mucosa for synthesis from free fatty acid. This could conceivably cause a delay in absorption.

Summary. From spectrophotometric examination of the tissue lipids of rats at 10, 16, 24 and 48 hours after being fed free conjugated linoleic acid or its glyceride, it is concluded that glycerides are absorbed more rapidly, and possibly more completely, than free fatty acids but that the pathways of absorption are the same.

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Competitive Elution of Pertussis Hemagglutinin. (18010)

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Keogh, North and Warburton(1) have observed that erythrocytes of various mammalian and avian species are agglutinated by saline suspensions of *Hemophilus pertussis* and related members of this genus. Hemagglutination was also induced by filtrates or cell-free supernatants of liquid-medium cultures of these organisms. Other investigators (2,3) have subsequently demonstrated similar hemagglutination with other bacterial species. Conditions for the production of the hemagglutinating substance in casein-hydrolysate broth, and the relation of the hemagglutinating substance to the antigen or antigens which induce protective antibodies against *H. pertussis* infection in mice, have been described by Fisher(4) and by Keogh and North(5) respectively.

In the course of a study of the behavior and properties of the pertussis hemagglutinin we attempted to elute the hemagglutinin from erythrocytes by various methods. Hemagglutination was obtained with the supernatants of 7- to 9-day-old cultures of virulent strains‡ of *H. pertussis* grown in Cohen and Wheeler's medium(6). The potency of such preparations was determined by adding

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5. Keogh, E. V. and North E. A., *Australian J. Exp. Biol. and M. Sc.,* 1948, v26, 315.

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0.5 ml aliquots of 0.5% suspensions of human red cells, to 0.5 ml volumes of serial 2-fold dilutions of *H. pertussis* supernatant, incubating at 37°C for 2 hours, and reading the agglutination pattern after the manner of Salk's technic for titration of influenza hemagglutinins(7).

Attempts to elute the hemagglutinating factor by varying the temperature of the mixture were unsuccessful. Agglutinated red cells were incubated at 37°C, 20-25°C or 4-6°C for as long as 3 days, and were then resuspended by agitation and allowed to settle. Agglutination was invariably re-established within 2 hours, with a pattern and a cohesiveness comparable to that originally observed.

In view of Lowell and Buckingham's report (8) that influenza virus could be eluted from erythrocytes by washing the cells in a 5% solution of glucose containing no added mineral salts, we attempted to elute pertussis-agglutinated erythrocytes in a similar manner. Following one washing of agglutinated cells with salt-free 5% glucose, the cells were resuspended in 0.9% NaCl solution; agglutination again readily reappeared.

It was observed (as Fisher(9) has independently noted) that the supernatant fluid from frozen and thawed red cells, resuspended in an equal volume of distilled water, was capable of blocking the agglutination of fresh red cells by *H. pertussis*. An experiment was therefore designed to determine the capacity of such a red-cell extract to elute the active substance from pertussis-agglutinated cells. Agglutinated cells were mixed with 5 volumes of a 1:5 dilution of red-cell extract in saline, shaken to resuspend the cells, and incubated at 37°C until the cells had settled. No hemagglutination appeared. Controls, consisting of agglutinated cells mixed with saline solution, showed normal reagglutination. The red-cell extract treated cells were then centrifuged, the supernatant discarded and the cells washed twice with normal saline solution. On adding fresh *H. pertussis* supernatant to the cells, hemagglutination reappeared after storage overnight at 4-6°C. To another aliquot of these cells, normal saline solution was added instead of *H. pertussis* supernatant; no hemagglutination appeared after storage in the cold.

Discussion. The failure of erythrocytes agglutinated by *H. pertussis* factor to resuspend, either after exposure to temperatures of 37°C or higher, or in the presence of lowered salt concentrations, is one of several features which distinguish this type of hemagglutination from that induced by the influenza-mumps-Newcastle disease group of viruses. Another distinction is found in the capacity of cell-free supernatants of *H. pertussis* suspensions to induce hemagglutination, in contrast with the influenza-mumps-NDV group. In both respects the reactions of the *H. pertussis* factor with susceptible erythrocytes resemble those exhibited by viruses of the pox group(10,11). Fisher(9) has also noted that *Vibrio cholerae* mucinase, which inactivates the cell receptor for influenza virus, does not affect pertussis hemagglutination.

However, hemagglutination by virus particles is not invariably followed by elution. It has been fairly conclusively demonstrated that, for influenza virus in particular, the power to elute runs closely parallel to the power to infect and to multiply; thus, influenza-virus particles, gently inactivated by heat, will adsorb to red cells but will not elute under the usual circumstances(12). On the other hand, influenza-virus particles, rendered non-infectious by brief ultraviolet irradiation, do not lose their capacity to elute unless the capacity to hemagglutinate has also been destroyed(13). The mechanism of attachment of the virus particle to the red cell appears therefore to be distinguishable from, although closely related to, the pre-

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13. Henle, W. and Henle, G., *J. Exp. Med.*, 1947, v85, 347.

sumably enzymatic property of the virus that leads in time to destruction of the receptor, and to elution. Thus the dynamics of hemagglutination by the *H. pertussis* factor, and by mildly inactivated influenza-type virus particles, may differ less markedly than would at first appear to be the case.

The observation that the bond between *H. pertussis* factor and red cells can apparently be dissociated in the competitive presence of red-cell extract, and that the cells thus released can again be agglutinated by addition of fresh *H. pertussis* factor, indicates that attachment of this factor to the red cell does not result in appreciable destruction of the receptor. The dissociation and reassociation reported here bears a closer resemblance to a familiar type of interaction which has been observed between many cellular antigens and their antibodies. Morgenroth, for example(14), noted that if sheep cells were saturated with hemolysin in the absence of complement, and then mixed and incubated with fresh cells for a certain minimum period, the addition of complement would then result in hemolysis of all the cells in the mixture. The influence of time, of amount of antibody added, and of the interaction of 2 red-cell species with an antibody active against both, have been studied in detail by Philosophow(15); and Mayer *et al.* (16) have recently confirmed and extended these observations. Another recent observation, parallel in principle, is that when Rh-positive red cells (presumably saturated with the corresponding maternally produced antibody) from an erythroblastotic infant are mixed with homologous Rh-positive adult blood, the cells of both infant and adult may hemolyze (17). Thus hemagglutination of the type induced by *H. pertussis* factor bears a limited resemblance not only to conventional virus hemagglutination, but also to conventional antigen-antibody interaction.

Summary. The hemagglutinating factor of *Hemophilus pertussis* culture supernatants can apparently be eluted from human erythrocytes by suspending such erythrocytes in a distilled-water extract of frozen and thawed red cells. Following the removal of the *H. pertussis* factor from agglutinated cells, these cells can be re-agglutinated by addition of fresh *H. pertussis* culture supernatant. The factor cannot be eluted by incubation of agglutinated cells at various temperatures, or by suspending the cells in salt-free isotonic glucose solution.

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Effect of 3-Hydroxy-2-phenylcinchoninic Acid on Renal Secretion of Phenyl Red and Penicillin.* (18011)

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Certain derivatives of cinchoninic acid have been found to be effective antidiuretics when administered to dogs with induced water diuresis(1,2), to cause an increased rate of excretion of uric acid when administered to man(3,4,5), to stimulate the anterior pituitary causing a decrease in the ascorbic acid content of the adrenal glands of rats(6), and to suppress the secretion of phenol red by the kidney due to blocking the tubular secretory mechanism(7,8). The present communication is concerned with this last pharmacological action, the blocking of renal tubular

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secretion. The effect on the secretion of phenol red is apparently similar to that described for carinamide(9).† Carinamide blocks the tubular secretion of phenol red and compounds such as p-aminohippuric acid and penicillin which are eliminated by the same tubular mechanism but does not interfere with the tubular secretion of N^1-methylnicotinamide(10). One should therefore expect that the derivatives of cinchoninic acid which suppress the secretion of phenol red would also suppress the secretion of penicillin. The present experiments confirm this expectation for 3-hydroxy-2-phenylcinchoninic acid (HPC).

All experiments were performed on female dogs. Suppression of the secretion of penicillin was estimated by determinations of its concentration in the plasma with and without previous injection of drug. The rate of excretion of phenol red was also measured with and without previous injection of drug. Crystalline potassium benzylpenicillin was given intravenously in a dosage of 2.4 mg per kg. Blood was drawn at one, 2, 3 and 4 hours after injection. Citrate was used as an anticoagulant, and the plasma taken for penicillin assay. Phenol red was injected intravenously in a dosage of 6 mg in 5 cc of saline. Urine was taken by catheter at one-half, one and 2 hours after injection, and its phenol red content determined. The drug was injected as the sodium salt intravenously 2 minutes before injection of the penicillin, or phenol red.

The concentration of penicillin in the plasma was determined by the method of Tompsett, Shultz and McDermott(11), using reconstituted lyophilized human plasma‡ as the diluent instead of pooled human serum. The organism used in the test was *Streptococcus dysgalactiae*§(12). The inoculum was made from a 10^{-2} dilution of a 24-hour subculture grown in trypticase soy phosphate broth. This inoculum was inhibited in the presence of 25% plasma by a penicillin concentration of 0.012 μg per ml. The end point in this titration was obtained by observing turbidity after 24 hours incubation at 37.5°C. The penicillin used for standards was crystalline potassium benzyl penicillin. A new standard was made up once weekly in a concentration of 300 μg per ml in distilled water and kept at 4°C. Fresh working standards were made daily from the concentrated standard, the last two dilutions being made up in 25% plasma. Recoveries from human plasma of added amounts (6.0 to 0.06 μg per ml) ranged from 80 to 100%.

The results of the experiments with penicillin injected alone, after carinamide, or after

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† In dogs, under conditions in which the extraction ratio of creatinine was around 20%, our method was not delicate enough to detect any difference between the concentrations of HPC in arterial and renal venous blood. The clearance of HPC in the dog is about 0.05 ml per minute; correcting for about 96% binding to plasma protein gives a clearance of unbound drug around 1 ml per minute. HPC does not appear to be secreted by the renal tubule.

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§ Dr. J. C. Kakavas of the University of Delaware kindly furnished us with the strain of *Str. dysgalactiae*.

TABLE I.
Effects of 3-Hydroxy-2-phenylcinchoninic Acid and Carinamide on Plasma Concentration of Penicillin in the Dog.

Dog	Hr after penicillin	Conc. of penicillin in plasma, μg per ml			
			Carinamide		HPC
		Control	20 mg/kg	40 mg/kg	20 mg/kg
1	1	2.8	3.0	4.8	5.6
	2	.8	.8	2.2	2.0
	3	.1	.2	.8	.8
	4	.0	.0	.3	.3
11	1	1.3	2.4	3.3	3.4
	2	.4	.6	.9	1.4
	3	.1	.3	.2	.6
	4	.0	.1	.0	.8
13	1	2.4		3.6	3.3
	2	.6		1.2	1.8
	3	.2		.7	.7
	4	.1		.3	.3

TABLE II.
Effects of 3-Hydroxy-2-phenylcinchoninic Acid and Carinamide on Urinary Excretion of Phenol Red in the Dog.

Dog	Hrs after phenol red	Mg of phenol red in the urine (cumulative)		
		Control	Carinamide 20 mg/kg	HPC 20 mg/kg
1	0.5	2.52	.71	.91
	1	3.52	1.17	1.58
	2	4.18	2.08	2.34
13	0.5	2.93	.86	.68
	1	3.82	1.36	1.69
	2	4.42	2.08	2.59

HPC are given in Table I. With the exception of the data on 20 mg per kg of carinamide where single experiments were done, the figures are averages of 2 or 3 experiments. It is evident from these results that the injection of 20 mg per kg of carinamide has no effect on the concentration of penicillin in the plasma, but that both 40 mg per kg of carinamide and 20 mg per kg of HPC give definitely higher concentrations of penicillin in the plasma. In Table II, the results of the experiments on phenol red excretion are given. The control values are the mean of 5 or 6 determinations which agree very closely. It can be seen from these data that 20 mg per kg of either carinamide or HPC markedly reduced the excretion of phenol red.

The effectiveness of carinamide and penicillin in elevating the concentration of penicillin in the plasma and in decreasing the excretion of phenol red is of the same order of magnitude. The data seem to indicate that HPC is more effective in raising the concentration of penicillin in the plasma than carinamide but this difference may not be significant.

We have interpreted our data to mean that HPC blocks the renal tubular secretion of both phenol red and penicillin. It has been shown that HPC decreases the renal clearance of phenol red without decreasing glomerular filtrate(8). This interpretation in the case of penicillin is based upon the assumption that the increase in the concentration of penicillin in the plasma is due to a blocking of the secretion of this substance by the renal tubular mechanism. However, there is every reason to believe that this assumption is correct. Carinamide has been found to

decrease the tubular secretion of penicillin and raise its concentration in the plasma after injection. The results reported indicate that cinchoninic acid derivatives which decrease the rate of excretion of phenol red will also block the renal tubular secretion of penicillin.

Summary. Similar to carinamide, 3-hydroxy-2-phenylcinchoninic acid causes higher concentrations of penicillin in the plasma when injected before the administration of the penicillin. Both substances also decrease the rate of excretion of phenol red. The activities of the two drugs are roughly of the same order of magnitude. This increase in concentration of penicillin in the plasma and decrease in rate of excretion of phenol red is attributed to a blocking of the renal tubular secretory mechanism.

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Desoxycorticosterone Acetate and Adenohypophyseal Content of Adrenocorticotrophic Hormone.* (18012)

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Twenty-four hours after adrenalectomy the content of adrenocorticotrophic hormone (ACTH) in the rat adenohypophysis is reduced to one-fifth of normal(1). Sayers and Cheng(1) favor the view that this marked depletion of pituitary ACTH is a consequence of rapid and uninhibited discharge of trophin in response to the absence of circulating cortical hormone. The data of the present paper are compatible with this interpretation. They demonstrate the desoxycorticosterone acetate (DCA) prevents the marked depletion of ACTH which normally occurs after adrenalectomy, and that DCA increases the concentration of ACTH in the adenohypophysis of the intact rat.

Methods. The donor rats were male animals obtained from the Sprague-Dawley farm. The body weights of these animals are presented in Table I. DCA pellets (six-15 mg pellets per rat) were implanted 72 hours before sacrifice and adrenalectomy was performed 24 hours before sacrifice. The animals were given free access to food and water at all times except those in Experiments 3 and 4, from whom food was withdrawn 24 hours previous to sacrifice. The animals were anesthetized with sodium pentobarbital, exsanguinated by severing the abdominal aorta and the pituitary dissected away from the sella. The anterior lobes were carefully freed of posterior pituitary tissue, frozen and lyophilized. The concentration of ACTH in the dried tissue was determined in hypophysectomized recipient rats by the method of Burns *et al.*(2).

A previous study(2) has demonstrated that each mg of dried adenohypophysis from untreated intact rats contains the biological equivalent of 80 μg of an ACTH standard (La-1-A†). This value was employed in the calculation of pituitary ACTH content. The weight of the pituitary tissue multiplied by ACTH concentration gives total hormone content per pituitary. For example, in Experiment 1 the content of ACTH in each pituitary of the intact untreated controls was obtained by multiplying 1.35 mg, the average weight of each pituitary, by 80 μg per mg, the concentration of ACTH in the tissue.

* Supported by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

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† Kindly supplied by Armour Laboratories.

TABLE I.
Effect of DCA on Pituitary Content of ACTH.

Exp. No.	Donor rats	No. donor rats	Avg body wt of donor rats, g	No. recipient rats	b	P	s	λ	M	Antilog M	S_M	Antilog (M — S_M) to antilog (M + S_M)	Pituitary dry wt, mg	ACTH per pituitary μg of La-1-A
1	intact DCA pellets	6	277	9	254	.2	42.7	.168	.132	1.36	±.090	1.10-1.67	1.25	136 (110-167)
	"	6	302	9	151		31.1	.206					1.35	108
2	" " "	6	328	12	73	.05-.02	23.9	.328	.154	1.42	±.101	1.13-1.80	1.57	179 (142-226)
	"	6	342	12	117		22.4	.191					1.57	126
3	sham adrenex	3	330	8	130	.7	29.7	.228	$\bar{1}$.861	.73	±.097	.58- .91	1.17	68 (54-85)
	intact	7	267	13	149		30.0	.201					1.19	95
4	adrenex	10	256	11	136	.7	24.4	.180	$\bar{1}$.306	.20	±.083	.17- .25	.98	16 (13-20)
	intact	7	267	13	149		30.0	.201					1.19	95
5	adrenex	6	297	9	171	.7	25.9	.151	$\bar{1}$.414	.26	±.084	.21- .31	1.16	24 (20-29)
	intact	6	302	9	151		31.1	.206					1.35	108
6	adrenex " "	6	289	9	180	.6	22.1	.123	$\bar{1}$.627	.42	±.078	.35- .51	1.19	40 (33-49)
	intact	6	302	9	151		31.1	.206					1.35	108
7	adrenex	6	238	11	191	.4	31.4	.165	$\bar{1}$.188	.15	±.130	.11- .21	1.08	18 (13-25)
	intact " "	6	247	10	103		8.6	.084					1.08	120
8	adrenex " "	6	253	8	75	.3	2.9	.038	$\bar{1}$.635	.43	±.059	.38- .50	1.15	55 (49-64)
	intact " "	6	247	10	103		8.6	.084					1.08	120

b = slope of the log dose response curve; P = probability factor measuring the significance of the difference between the slopes; s = standard deviation of all of the individual responses about this curve (a straight line fitted by the method of least squares); λ = s/b, a measure of the accuracy of the assay method; M = logarithm of the ratio of the potencies; S_M = standard error of M; antilog (M — S_M) to antilog (M + S_M) is the range of estimate for one standard error.

Each of the pituitaries of intact animals treated with DCA had an ACTH content equal to 1.25 mg, the average weight of each pituitary, times 80 μg per mg multiplied by 1.36, the ratio of potencies of the DCA-treated to the untreated animals.

Results. The assay data of the various experiments are presented in Table I. For a detailed discussion of the statistics the reader is referred to the paper of Sayers *et al.*(3). Suffice it to point out here that the antilogarithm of M gives an estimate of the ratio of potencies of the assay pairs. The calculation antilogarithm $(M - S_M)$ to antilogarithm $(M + S_M)$ is the range of the estimate for $P = 0.67$.

In Exp. 1 and 2 (Table I) the influence of DCA upon the concentration of ACTH in the adenohypophysis of the intact rat was determined. In both experiments DCA induced a small but significant increase in both the concentration and content of adenohypophyseal ACTH. In Exp. 2, for an unexplained reason, the slopes derived from the data of the two members of the assay pair appear to be different. The P value for the significance of the difference of the slopes is 0.05 to 0.02.

Sham adrenalectomy (Exp. 3), performed 24 hours before sacrifice, resulted in a significant reduction (27%) in the concentration of pituitary ACTH. Bilateral adrenalectomy induced a very marked depletion of adenohypophyseal stores of ACTH. In Exp. 4, an 80% and in Exp. 5, a 74% reduction in hormone concentration occurred 24 hours after this operation.

Exp. 5 and 6 were conducted simultaneously. The concentration of ACTH in the pituitaries of the untreated adrenalectomized animals of Exp. 5 and that of the DCA-treated animals of Exp. 6 were each compared with the ACTH stores in the same untreated intact controls. DCA appreciably inhibited the marked reduction in pituitary concentration of ACTH which normally follows adrenalectomy. However, the steroid did not restore the value to that of intact sham-operated animals.

In Exp. 7 and 8, ACTH stores in untreated and DCA-treated adrenalectomized animals were compared with those in DCA-treated intact rats. The results confirm those of Exp. 5 and 6. DCA actually increased pituitary ACTH content in adrenalectomized rats to a value not significantly less than that in sham-operated intact animals.

Discussion. DCA induces adrenal cortical atrophy(4-8) and prevents the reduction in adrenal ascorbic acid which normally occurs when the rat is subjected to acute stress(9). These observations suggest that the steroid inhibits discharge of ACTH from the adenohypophysis. The data of the present report demonstrate that DCA increases the ACTH content of the pituitary in intact rats and inhibits the depletion of pituitary stores of ACTH which normally follows adrenalectomy. The results are compatible with the concept that DCA depresses the rate of discharge of ACTH from the adenohypophysis. However, they do not constitute proof of such a concept. It is to be emphasized that pituitary content of hormone is not necessarily related to rate of discharge of hormone. (For an analysis of this problem, see the paper by Sayers and Cheng(1).) However, it has been demonstrated by Taylor *et al.*(10) that the blood of patients with untreated Addison's disease, but not that of normal subjects, contains detectable quantities of ACTH.

DCA in the doses employed in these experiments allows some depletion of pituitary ACTH stores in adrenalectomized rats even when the comparison is made against the hormone content of the pituitaries of sham-operated animals. It would be of interest to know whether larger doses of DCA bring about complete inhibition of pituitary loss of

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ACTH. Additional studies employing various doses of both DCA and cortisone are contemplated.

The metabolic and toxic actions of DCA in the intact organism are a result, in part at least, of its inhibitory effect upon pituitary adrenocorticotrophic activity. The steroid induces insulin hypersensitivity(11). Furthermore, the elevation of electroshock threshold and hypernatremia, normally produced by DCA, are prevented by the simultaneous administration of ACTH(12).

In addition to a pituitary inhibitory effect, it is quite probable that DCA induces certain changes characteristic of adrenocortical insufficiency by competing with cortisone-like compounds for strategic loci in peripheral effector cells. This aspect of the problem is undergoing study in our laboratory at the present time.

The data of the present report have an important bearing on the mechanism of action of DCA in inducing pathological changes in experimental animals similar to those observed in the collagen diseases in man. It has been suggested by Cheng and Sayers(11) and by Woodbury *et al.*(12) that this toxic action of DCA is a result, in part, of a steroid hormone imbalance, namely, an excess of DCA (exogenous synthetic steroid) and a deficiency of the secretion of the adrenal cortex. The recent demonstration of Woodbury *et al.*(13) that ACTH given simultaneously with DCA can inhibit the development of pathological changes, which are normally induced by the synthetic steroid when administered alone, lends strong support to this interpretation.

Summary. DCA pellets increase the concentration of ACTH in the adenohypophysis of the intact rat. The steroid inhibits the marked depletion of pituitary stores of ACTH which normally occurs 24 hours after adrenalectomy.

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Cortisone Acetate and Terramycin in Polyarthritis of Rats.*† (18013)

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The L_4 strain of pleuropneumonia-like organism will produce in rats an arthritis which has been used as a method of evaluating various therapeutic agents(1-5). Pleuropneumonia-like organisms are evidently species specific as far as pathogenicity is concerned so that human strains cannot be used in animals. Dienes and his associates

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† The cortisone for this study was allocated by the Committee on Cortisone of the National Academy of Science, and the terramycin was the gift of the Charles Pfizer Co.

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TABLE I.
Preventive Effect of Cortisone in Polyarthritis of Rats.

Exp. No.	No. of rats	No. of inj.*	Dosage (mg/kg)	Avg max arthrogram score	Incidence of arthritis, %	Survival rate, %
V	20	14	2.5	1.3	70	85
	20	control	—	1.7	60	60
VI	18	13	2.5	4.0	89	66
	17	control	—	3.5	70	88
X	20	14	20.0	2.5	90	65
	20	control	—	2.0	70	100

* Injections were given once daily beginning on the day of infection.

have repeatedly isolated such organisms from the human genitourinary tract and feel that they "may be related etiologically to an acute infectious type of arthritis and to Reiter's syndrome(6)." Brown and his group have reported on the favorable effects of aureomycin treatment of rheumatic patients harboring pleuropneumonia-like organisms(7). In this laboratory human strains of pleuropneumonia-like organisms have been isolated from conjunctivae, urethral discharge, penile lesions, and joint fluid in 5 cases of Reiter's syndrome, and there has been one case of cross-infection which is to be separately reported.

In the polyarthritis of rats the use of gold salts will either prevent the development of the arthritis or will promote more rapid healing if given after the joints are swollen. Since gold salts often favorably influence the clinical course of rheumatoid arthritis in man various compounds have been tried in this rat infection in an effort to find agents which have the same therapeutic effect, but with less toxicity, for possible therapeutic trials in man.

Since cortisone acetate (Kendall's Compound E or 11-dehydro-17-hydroxycorticosterone-21-acetate) has produced such striking alterations in the clinical status of patients with rheumatoid arthritis(8) an attempt has been made in this study to evaluate its preventive and therapeutic properties in the polyarthritis of rats which is a specific infection. Terramycin has been evaluated in this study since aureomycin was shown to be effective in a previous study of the polyarthritis of rats(3).

Methods. Cortisone was given to the infected rats by daily intramuscular injection. The cortisone was prepared by Merck and was supplied in a saline suspension containing 25 mg per cc. The controls received daily intramuscular injections of the same volume of physiological saline solution. The terramycin used was the commercial product of the Charles Pfizer Co. It was administered by mixing with the diet (ground Purina laboratory chow), in the drinking water, and by stomach tube. The criteria used in evaluating these drugs included the per cent incidence of arthritis in each group, the extent of the joint involvement scored numerically, the per cent survival rate, and in some instances the per cent weight gain during the experimental period. The extent of the joint involvement was evaluated according to a modification of the arthrogram of Sabin and Warren(9) which assigns a numerical value of 4 to each front leg and 5 to each hind leg, giving a total of 18 points per animal assuming maximum joint involvement of all joints of the extremities. The average arthrogram scores were determined by adding the highest scores for each individual animal and dividing the totals by the number of animals in the group irrespective of per cent incidence of arthritis.

The microbe was cultured in a broth which

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was prepared as follows, per liter distilled water: 3 g yeast extract, 7 g nutrient broth concentrate, 10 g tryptose phosphate concentrate (Difco), and 2 g dextrose, C.P. For solid media 2% agar agar and for broth 0.75 g agar agar per liter were added. The pH was adjusted as follows before autoclaving: for solid agar to 8.23, and for broth to 8.1. The media was then sterilized by autoclaving at 15 lb pressure for 20 minutes. Sterile, Seitz-filtered horse serum was then added aseptically in the concentration of 20%.

Albino rats were used in all experiments with an equal number of males and females in each group. The weight of the animals at the time of infection was between 60 and 100 g. The infection was induced by the intraperitoneal injection of 2 cc of a broth culture of the microorganism. This injection was a mixture of equal parts of 24- and 48-hour cultures of pathogenic strains. Since the arthritic pathogenicity of strains varies considerably it was necessary in each instance to run separate controls.

Results. Cortisone. Table I shows the preventive effect of cortisone administered once daily beginning on the day of infection. The average maximum arthrogram scores show that in one experiment the joint involvement was slightly less and in the other slightly more than in the control groups when the cortisone dosage was 2.5 mg per kg. Likewise, in one experiment at this dosage the per cent incidence of arthritis was less and in the third group more, and the per cent survival rate showed the same inverse relationship in the latter 2 groups. The dosage of 20 mg per kg in one experiment was accompanied by a slightly more extensive joint involvement, increased incidence of arthritis, and a decreased survival rate.

Table II shows the evaluation of the therapeutic effect of cortisone in 8 separate experiments. The medication was begun 6 to 8 days following the infection and at a time when the involved joints were beginning to swell. Five of the experiments were run with a daily dosage of cortisone of 2.5 mg per kg. In these groups generally the per cent incidence of arthritis was less in the controls than in the medicated animals at the

TABLE II. Therapeutic Effect of Cortisone in Polyarthritis of Rats.

Exp. No.	No. of rats	No. of inj.*	Dosage (mg/kg)	Incidence of arthritis (%)		Avg arthrogram score		Survival rate, %	Avg weight		Wt gain during treatment, %
				Beginning of treatment	End of treatment	Beginning of treatment	End of treatment		Beginning of treatment, g	End of treatment, g	
II	40	10	2.5	35	23	0.7	.38	98	87	118	36
	19	control	—	32	16	0.5	.1	100	88	126	43
III a	10	15	2.5	60	20	1.1	.4	100	70	107	53
	9	control	—	44	22	1.1	.3	100	80	133	66
b	10	15	2.5	70	20	1.9	.2	100	73	113	55
	9	control	—	56	22	0.7	.2	100	76	121	60
IV a	9	16	2.5	56	44	1.7	.66	89	80	114	43
	9	control	—	56	33	0.5	.33	100	72	111	54
b	9	16	2.5	67	33	1.7	.33	100	77	126	64
	9	control	—	67	11	1.0	.22	78	67	119	78
VII	19	13	10	89	84	2.4	1.8	95	76	84	11
	19	control	—	63	79	2.0	2.7	100	78	106	36
IX a	9	14	10	78	78	2.7	2.8	100	88	97	10
	9	control	—	89	77	2.4	2.3	89	99	129	30
b	8	14	10	75	63	3.0	2.5	63	61	64	5
	10	control	—	78	78	1.9	3.6	78	60	73	22

* Injections were given once daily beginning 6 to 8 days following infection.

TABLE III.
Therapeutic Effect of Terramycin in Polyarthritis of Rats.*

No. of rats	Dosage	Incidence of arthritis (%)		Avg arthrogram score		Survival rate, %
		Beginning of treatment	End of treatment	Beginning of treatment	End of treatment	
19	0.3% in drinking water 4 days	68	47	1.9	0.8	100
20	Control	75	80	2.7	3.2	90

* Medication began 8 days following infection.

TABLE IV.
Preventive Effect of Terramycin in Polyarthritis of Rats.*

No. of rats	Dosage	Avg max. arthrogram score	Incidence of arthritis, %	Survival rate, %
18	50 mg/kg stomach tube 2 days	.11	5.5	67
10	0.1% diet 12 days	.5	40	100
10	0.3% diet 12 days	.0	0	100
20	Control	3.7	80	75

* Medication began on the day of infection.

end of treatment. The extent of the joint involvement was about the same. In one experiment the survival rate was less in the controls and in 2 others it was slightly less in the medicated animals. The remaining 2 experiments showed 100% survival of both groups. The per cent weight gain was less in the medicated rats than in the controls in these 5 experimental groups. At a dose of 10 mg cortisone per kg the per cent incidence of arthritis remained the same in one experiment and decreased slightly in 2 other groups during the period of medication. In their controls one group remained the same, one decreased slightly, and one increased slightly. The extent of joint involvement decreased in 2 and increased slightly in one of the medicated groups while in their controls the arthritis became more extensive in 2 groups and decreased slightly in another. The survival rate was slightly less in 2 and greater in one of the treated groups as contrasted with their controls. The per cent gain in weight was less in the medicated animals as compared with controls in each of the 3 groups on the 10 mg per kg dosage schedule.

Terramycin. Table III demonstrates the therapeutic effect of terramycin given for 4 days in the drinking water to the animals 8 days following infection. The incidence of joint arthritis decreased 21% in the medicated animals as compared with 15% in the controls; whereas, the extent of joint involvement decreased more than half in the treated while in the controls it increased slightly. All treated animals survived while 10% of the controls died. The addition of 0.3% terramycin to the ground food at the time of infection prevented the development of arthritis and permitted 100% survival as compared with 80% incidence of arthritis and 75% survival rate in the controls (Table IV). With 0.1% terramycin in the diet only half as much arthritis developed in the treated animals as in the controls and all survived. When terramycin was administered by stomach tube for 2 days beginning on the day of infection only 5.5% developed arthritis as compared with 80% of the controls. While 67% of those medicated via stomach tube survived as compared with 75% survival among the controls the difference may have been due to trauma incident to intubation. The controls were used for the other 2 groups as well and therefore were not intubated with a placebo material.

Discussion. Cortisone apparently does little to alter the course of the rat polyarthritis when it is used as a preventive agent, and when used therapeutically the differences were also small. Generally speaking the medicated animals looked worse at the end of the experiments and weighed less than the controls. Selye has reported that cortisone and ACTH

will inhibit in albino rats the development of arthritis after injection of formalin into the joints(10). We have previously reported that ACTH lacked a beneficial effect as a preventive in the polyarthritis of rats(4). Since there is no exact counterpart in experimental animals of human rheumatoid arthritis, it is interesting that in these 2 types of experimental arthritis the results with cortisone and ACTH are different. It is also of interest that gold salts will favorably influence the rat polyarthritis as well as many cases of rheumatoid arthritis in man. It would be interesting to check the use of gold salts in the formalin arthritis.

In view of the previous reports of the beneficial effect of aureomycin on pleuropneumonia infections in animals and man (3,7) it is interesting that terramycin is also effective in preventing the development of the rat polyarthritis. This finding suggests that terramycin might be given clinical trial in patients with Reiter's syndrome and in early cases of rheumatoid arthritis.

Summary. Cortisone failed to prevent the development of the polyarthritis of rats when its administration was begun on the day of infection or to improve it when given therapeutically. The animals receiving cortisone gained less weight than the unmedicated controls. Terramycin given in 0.3% in the diet prevented the development, and 0.3% in the drinking water favorably altered the course of the rat polyarthritis.

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Blood and Packed Cell Volume of the Adult Rat as Measured by Tagged Cells.* (18014)

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The blood volume of the rat derived from the dilution of intravenously administered dyes by plasma has been reported frequently. Estimates vary from about 4.3 to 8.0 ml per 100 g body weight(1-8), with a mean of approximately 7.0 ml. Berlin *et al.*(9), on the basis of the dilution of transfused blood containing cells tagged with radioactive iron, phosphorus, or both, reported a mean volume of about 4.6 ml per 100 g, a value considerably lower than most obtained by the plasma-dye method.

It seemed pertinent to measure adult rat blood and packed cell volumes employing both the transfused radio-iron tagged erythrocyte and hemoglobin extraction(10) technics and to compare these data with those of Berlin (9). The tagged cell transfusion method was also applied to the evaluation of the efficiency of perfusion with respect to the total rat as well as individual tissues.

Experimental. The rats used were adult males of the Sprague-Dawley strain bred in this laboratory and maintained on a Purina ration. They appeared in no way unusual.

* Research carried out at Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

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TABLE I.
Blood and Packed Cell Volumes of 16 Adult Male Rats as Measured by Transfused* Tagged Cells.

Wt, g	Blood vol., ml/100 g body wt	Hct.	Packed† cell vol., ml/100 g body wt
330	4.64	.46	2.07
340	5.15	.49	2.47
331	4.83	.48	2.25
346	4.88	.51	2.43
340	5.00	.46	2.24
321	4.92	.52	2.49
312	4.90	.48	2.28
330	5.33	.45	2.33
330	4.79	.49	2.28
308	4.77	.49	2.27
260	4.19	.47	1.89
300	5.10	.49	2.43
360	5.37	.50	2.55
342	4.97	.50	2.34
322	5.40	.48	2.44
350	5.30	.48	2.40
Mean	4.98		2.32
Stand. Dev., %	6.3		7.3

* Rats 1-12 inclusive were transfused with 0.5 ml of blood containing Fe^{59} tagged cells; rats 13-16 inclusive with 1.0 ml of blood containing Fe^{55} tagged cells.
† Corrected for transfused cells.

Donors of blood cells labeled with radioactive iron were prepared by intraperitoneal injection of 1-2 ml ferric ammonium citrate solution, pH 7, containing about 0.5 mc Fe^{59} or Fe^{55} per mg iron.† Approximately 10 days later the animals were anesthetized with ether and bled maximally from the abdominal aorta, heparin being used as anticoagulant. The radioactivity of the blood was confined practically completely to the cells and was at a maximum at this time. Before transfusion, the blood of most recipients was tested for compatibility with that of their respective donors. No evidence of incompatibility was observed.

Under ether anesthesia, a group of 16 rats was transfused by way of the exposed femoral or epigastric vein; the first 12 received 0.5 ml of Fe^{59} tagged blood and the remaining four 1.0 ml of Fe^{55} tagged blood. After about 2 hours, again under anesthesia, the recipients were bled from the abdominal aorta or inferior vena cava. The radioactivity of blood from donors and recipients was assayed(11) using argon filled (76 cm) G-M counters that responded to both the Fe^{59} beta rays and Fe^{55} X-rays. The initial sampling of donor blood, carried out immediately following the transfusions, was made with the same syringe and attached needle used for injection of the blood, the volumes measured being the same as those transfused. The maximum difference in radioactivity between any two of quadruplicate samples of donor blood and between duplicate samples of recipient blood was about 2%. The reproducibility of the measurements appears sufficient to rule out any considerable error from these sources. The fraction of packed cells in the samples of blood, *i.e.*, the hematocrit, was determined in duplicate using Wintrobe tubes. Centrifugation was carried out at 3000 rpm for 30 minutes; the distance from the center of the rotor to the bottom of the tube was 22.2 cm.

The ratio of the radioactivity transfused to the activity per unit volume of recipient's blood expresses the blood volume of the recipient. This value multiplied by the hematocrit gives the volumes of packed cells. Rat No. 15 (Table I) received the highest ratio of

† Obtained as the chloride from the Isotopes Division, U. S. Atomic Energy Commission. The two radioisotopes of iron were prepared by neutron bombardment of enriched Fe^{54} and Fe^{58} in a nuclear reactor.

TABLE II.
Blood and Packed Cell Volumes of 6 Rats Both by the Transfused Tagged (Fe^{55}) Cells and Hemoglobin Extraction Methods.

	ml of blood per 100 g body wt			ml of packed cells per 100 g body wt	
	Tagged cells	Hb extraction	Hct.	Tagged cells*	Hb extraction*
	5.06	5.10	.49	2.48	2.50
	4.96	4.85	.50	2.48	2.43
	4.90	4.92	.44	2.16	2.16
	4.68	4.61	.46	2.15	2.12
	4.83	4.93	.46	2.22	2.26
	4.90	5.09	.48	2.35	2.44
Mean	4.89	4.92		2.31	2.32

All rats were transfused with 0.75 ml of donor blood.
* Corrected for transfused cells.

transfused blood to body weight. Consequently, the transfused blood might have increased the blood volumes by as much as six percent. The blood volumes reported are those actually found since the fraction of transfused plasma remaining in the circulation after 2 hours is uncertain. On the other hand, the packed cell volumes have been adjusted for the transfused cells. The final blood and packed cell volumes are given in Table I. The volumes found are in good agreement with those previously obtained using the labelled-cell method in another strain of rat(9), the latter values being about 4.6 and 2.2 ml per 100 g for the blood and cell volumes respectively. The agreement is somewhat closer than appears usual when the plasma-dye method has been used in the rat by different workers.

Blood and cell volumes were similarly determined in another group of 6 rats, 0.75 ml of labeled (Fe^{55}) blood being transfused. In these same animals, the volumes were also estimated by comparison of the concentration of hemoglobin in blood removed from the animals with that remaining in the carcasses(10). The volumes found are given in Table II. The results given by the two methods in this group are in good agreement with each other and also agree with those found in the first group (Table I). The mean blood and packed-cell volumes and their standard deviations for all 22 of the rats using labeled-cells are $4.95 \pm .27$ and $2.32 \pm .16$ ml per 100 g body weight respectively.

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Although the labeled-cell procedure indicates a blood volume in the rat lower than most furnished by the plasma-dye method, it seemed pertinent to test whether significant amounts of the injected blood were removed from the circulation during the interval between injection and final collection of blood from the recipients. Such removal would lead to a higher apparent volume.

Thus a number of rats were transfused as indicated previously, then bled maximally from the inferior vena cava. Ringer's solution was then introduced through the needle used in withdrawing the blood until the heart stopped beating vigorously. To improve heart action at this time the needle was transferred to the right auricle and perfusion continued. The perfusion fluid and blood were permitted to drain from the inferior vena cava about an inch below the needle. Removal of blood from the viscera was aided by occasional massage. At the end of perfusion, individual tissues and the remainder of the carcass were assayed for radioactivity‡ and the presump-

‡ In the determination of radioactivity an appropriate amount of inert iron is mixed with a digest of tissue; the tissue iron, of which some is radioactive, and carrier iron are collected by treatment of the mixture with ammonium hydroxide; the iron is then plated on copper disks(11). The presence of relatively large amounts of salts from tissues interferes with plating. In some cases treatment of the ammoniacal mixture of digest and carrier iron with hydrogen sulfide yields a product that plates more readily. In extreme cases purification of the iron precipitated by ammonia or sulfide by means of cupferron has been necessary.

TABLE III.
Blood Volume of Tissues of 4 Rats Following Perfusion with Ringer's Solution Measured by Transfused Tagged (Fe^{55}) Cells.

Estimate of perfusion	ml of blood per 100 g of tissue						
	Heart	Kidney	Brain	Spleen	Leg muscle	Liver	Carcass
Excellent	.062	.208	.014	11.5	.081	.910	.172
"	.040	.095	.024	11.1	.082	.296	.244
"	.132	.132	.008	10.8	.049	.456	.231
"	.031	.089	.007	9.94	.046	.347	.264
Mean	.066	.131	.013	10.8	.065	.502	.228

TABLE IV.
Blood Volume of Tissues of 7 Rats Following Maximal Bleeding, Measured by Transfused Tagged (Fe^{59}) Cells.

	ml of blood per 100 g of tissue					
	Heart	Kidney	Brain	Spleen	Leg muscle	Liver
	4.0	9.0	1.0	10.3	0.6	7.7
	3.9	5.8	0.7	16.0	0.4	4.1
	5.3	2.5	0.8	12.8	0.3	4.4
	4.1	3.0	0.8	11.7	—	4.0
	3.8	5.1	0.7	27.7	0.3	4.5
	3.9	3.6	0.6	16.3	0.3	3.4
	4.1	5.4	0.7	21.6	0.4	5.1
Mean	4.2	4.9	0.8	16.6	0.4	4.7

tive blood volumes calculated.

The data for individual tissues are given in Table III; the total rat blood volumes, before perfusion, are given in Table I (Rats 13-16). The apparent volume of blood remaining in the whole animal after perfusion was about five percent of the volume found in the same non-perfused rat. Thus, the apparent blood volume indicated by the labeled-cell procedure may be as much as 5% too high.

For comparative purposes and to evaluate the efficiency of the perfusion, the blood volumes of individual tissues from rats bled maximally were determined by means of transfused labeled cells. The data are given in Table IV. It appears that the perfusion removed an appreciably smaller amount of the radioactivity, about 30%, from spleen than from other tissues. The radioactivity apparently represented blood and not deposited iron since the average blood volume of four spleens, taken from perfused rats and assayed by the hemoglobin method, was 11.7 ml per 100 g of body weight.

Discussion. Tagged cell methods employed by us measure packed cell volumes. If the average body hematocrit is known, these methods may be used to measure blood volumes. The large vessel hematocrit may differ from that of the average body hematocrit although such a difference has not been reported for the rat. Gibson(12) found the average body-hematocrit to be 9 percent lower than that of the large vessels in humans and dogs. Mayerson(13), using humans, concluded that the difference between the two hematocrits is negligible. If one assumes this difference to be 9% in the rat, then the blood volumes reported here are 9% too low. The hemoglobin assays are subject to a further correction for extracted myoglobin that may be of the order of 3%(14).

In view of the wide variation in the re-

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ported rat blood volumes obtained by the dye method, it is not possible to draw any conclusions as to the factors responsible for the difference between these data and that yielded by transfused tagged cells.

Summary. 1. The mean blood and packed cell volumes of 22 adult male rats, as measured by transfused tagged cells, was found to be 4.95 and 2.32 ml per 100 g of body weight respectively.

2. The mean blood and packed cell volumes of six of the 22 rats, assayed by the hemoglobin extraction method, were found to be 4.92 and 2.32 ml per 100 g of body weight.

3. The efficiency of perfusion for the total rat was 95%. This corresponds to a possible error in blood volume, introduced by removal of tagged transfused cells from the recipients circulation, no greater than 5%.

4. Comparison of the blood volume of various tissues taken from maximally bled rats with the blood volumes of similar tissues taken from perfused rats indicates that perfusion is least effective for spleen and most effective for kidney and brain.

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Isoimmunization in the Pig Following Multiple Transfusions of Incompatible Blood.* (18015)

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The existence of 4 distinct blood group systems in the pig has been described(1). These have been conveniently designated as Pi groups 1, 2, 3 and 4 (Pi_1, Pi_2, Pi_3, Pi_4). The Pi_1 group gave the consistently strongest reactions and was dominant in this respect over the other 3 groups. Members of Pi_1 possessed either the homologous antigen or antibody, or, in 15% of animals studied, carried neither antigen nor antibody (O_1). Members of Pi_2 were similar in this respect except that (O_2) was more common than the homologous antigen or antibody (77.9%). There was a reciprocity between the Pi_1 and Pi_2 antigen-antibody components when they occurred together. Occasionally an animal was O for both groups. Further isoagglutination and absorption experiments demonstrated the existence of two additional reciprocally related systems (Pi_3 and Pi_4) which were somewhat analogous to the A and B blood groups in man. On the basis of these groups, it was possible to predict the existence of fourteen blood group combinations.

In the present report, 2 pigs of anti-Pi_1 specificity were injected with serial blood transfusions from one pig of Pi_1 specificity over a period of 43 days in order to determine the immunizability of the recipient pigs against this blood group antigen. This was done with a view to utilization of the pig as an experimental animal in the production of experimental hemolytic syndromes. Young(2) has reported similar procedures in the dog.

Methods. The schedule of isoimmunization was so arranged that citrated whole blood from the donor pig (Pi_1) was given intravenously by syringe to 2 recipient pigs (anti-Pi_1) in amounts which increased progressively from 7.5 ml to 15.0 ml every 2 to 3 days over a period of 26 days. Following this time, the quantity of blood given at any one time was

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2. Young, L. E., Ervin, D. M., Christian, R. M. and Davis, R. W., *Science,* 1949, v109, 630.

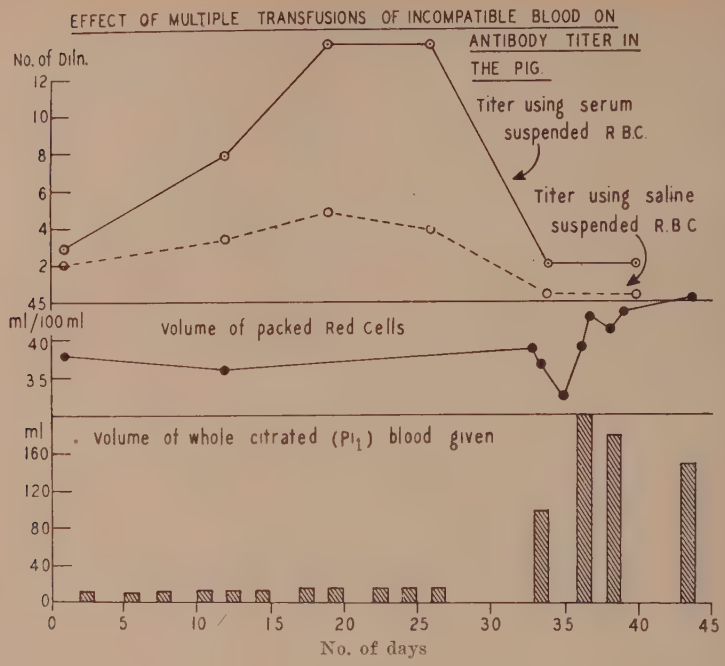

FIG. 1.

increased, so that amounts which varied from 100 to 200 ml were given in a total of 4 infusions over an 18 day period. Frequent whole blood samples were withdrawn for the determination of the volume of packed red cells, icterus indices and antibody titrations. All titrations were performed utilizing both saline and "blocking" technics. Determinations of "blocking" antibodies were made by serially diluting the recipient pigs sera (anti-Pi_1) with pooled Pi_1 sera, and adding equal amounts of serum suspended Pi_1 red cells to each dilution. Saline titrations were performed in similar fashion with the exception of the change of diluent.

Results. The results in one recipient pig are indicated in Fig. 1. Analogous results were obtained in the second transfused pig. There occurred no consistent fall in the volume of packed red cells during the total period of isoimmunization, nor did the icterus indices rise to abnormal levels at any time. Although titrations of the sera for anti-Pi_1 antibodies showed no demonstrable increase in titer in saline suspended Pi_1 red blood cells during the total period of study, the titer of "blocking" antibodies of anti-Pi_1 specificity in both animals increased to a marked extent during the same time. However, following the infusion of large quantities of blood the titers practically disappeared, along with a transient fall, followed by a progressive increase in the volume of packed red cells.

Discussion. It was of interest that isoimmunization of 2 pigs against a naturally occurring blood group antigen (Pi_1) resulted in the formation of "blocking" or incomplete antibodies of high titer against Pi_1. The sera of both animals prior to transfusion yielded approximately the same low titers in both serum and saline suspended cells, which were interpreted as indicating the presence of naturally occurring anti-Pi_1 agglutinins. However in the course of the Pi_1 transfusions there appeared the so-called "immune" anti-Pi_1 antibodies of the "blocking" variety in high titer (demonstrable in serum suspended Pi_1 red cells) while the saline titers showed no appreciable change (Fig. 1). This situation is some-

what analogous to the findings of Witebsky (3), Ervin and Young(4) and Wiener(5) in man where incompatible transfusions or heterospecific pregnancies involving the A-B-O blood groups occasionally result in the formation of "blocking" or incomplete antibodies against blood groups A or B. The "blocking" antibody in turn is of importance in the pathogenesis of erythroblastosis fetalis and in transfusion reactions.

Whether selective fetal death in the pig is ever the result of transplacental isoimmunization and the formation of "blocking" antibodies, is unanswerable at the present time. The studies of Corner(6), however, may be pertinent in this regard, and his concept of embryonic morbidity in mammals as a sequel to internal defects of the germ cells may profitably be re-investigated in the present light of demonstrable blood group incompatibilities in many mammals, including the pig.

Hemolysis in the transfused pigs was apparently not great as judged from the serial icterus indices and volume of packed red cell determinations. However, the initial decrease in volume of packed cells which took place shortly after the titer decrease suggests a possible transient hemolytic episode because of antibody absorption by Pi_1 red cells. This point will require further investigation in view of the fact that Ashby studies were not performed, and urobilinogen excretion in urine and stools was not determined.

That hemodilution was the most likely basis for the sudden titer decrease is suggested by the fact that blood typing of the anti-Pi_1 pigs subsequent to the transfusion of large volumes of Pi_1 blood revealed considerably weaker agglutination than was present prior to the experiment. In addition, there occurred a progressive concomitant rise in the volume of packed red cells after the initial temporary fall.

Summary. Multiple transfusions of Pi_1 blood from one pig into two other pigs of anti-Pi_1 specificity resulted in the formation of high titers of "blocking" antibodies of anti-Pi_1 specificity. The implications in experimental hemolytic syndromes and in selective fetal death in the pig are discussed.

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An Improved Assay for "Strepogenin" Based on Essential Nature of Material for *Lactobacillus bulgaricus* 09. (18016)

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"Strepogenin" has been applied as a name to a factor or group of closely related factors essential for the growth of certain Group A streptococci(1,2) or stimulatory for the growth of a variety of lactic acid bacteria (2-4). Partial hydrolysates of certain purified proteins have been shown to have high "strepogenin" activity(2,4,5). Recent work has suggested that the amino acids serine,

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glycine, and glutamic acid might be involved in the "strepogenin" structure(6,7). It appears possible that a variety of "strepogenins" exist, having one parent or basic structure to which other amino acids are combined.

Although "strepogenin" is an absolute growth factor essential for some of the Group A streptococci, growth obtained with these strains is not abundant. Many investigators have resorted to the use of an assay based on growth stimulation of *Lactobacillus casei* for the estimation of "strepogenin" activity. The response of *Lactobacillus casei* to "strepogenin" is not specific, however, since it has been shown that under defined conditions asparagine and glutamine as well as a variety of synthetic peptides have "strepogenin" activity(6-10).

Investigations on the nature of an unknown growth factor required by *Lactobacillus bulgaricus* 09 have shown that the growth-promoting activity of natural materials can be duplicated with synthetic orotic acid(11). The basal medium used in these studies contained, in addition to the usual components of a semi-purified medium, norit-treated tryptic digest of casein, yeast extract, and vitamin B_{12}. When it became possible to grow *Lactobacillus bulgaricus* 09 with orotic acid in this medium, studies were undertaken to determine the extent to which the norit-treated tryptic digest of casein, the yeast extract, and vitamin B_{12} contributed to the growth factor requirements of the organism. It was found that the yeast extract and the vitamin B_{12} were dispensable components of the medium provided adequate amounts of the norit-treated tryptic digest of casein were present. Studies on the nature of the growth factor contributed by the norit-treated tryptic digest of casein have shown that the material has the properties and distribution of "strepogenin." Under the conditions to be described essentially no growth, even on prolonged incubation, is obtained with *Lactobacillus bulgaricus* 09 in the absence of "strepogenin." In the presence of adequate amounts of "strepogenin", however, good turbidity and high acid production result.

"Strepogenin" has not yet been isolated and identified. One reason for lack of progress undoubtedly lies in the non-specific nature of the *Lactobacillus casei* assay usually employed. The use of *Lactobacillus bulgaricus* 09 appears to offer definite advantages over the *Lactobacillus casei* procedure and may be of aid to those concerned with the isolation or biochemistry of "strepogenin."

Procedure. The organism employed was *Lactobacillus bulgaricus* 09 of the Cornell collection.* The organism was maintained by daily transfer in sterile skim milk supplemented with 1% tryptose (Difco). The incubation temperature was 37°C. Inocula for seeding microbiological assays were prepared

TABLE I.
Composition of Double Strength Basal Medium.*

Acid-hydrolyzed norit-treated vitamin-free casein	1.0	g
Glucose	4.0	"
Sodium acetate (anhydrous)	1.2	"
L-Tryptophane	20	"
L-Cystine	20	"
Orotic acid	4	"
Adenine	1	"
Guanine	1	"
Xanthine	1	"
Uracil	1	"
Salts A	1	ml
Salts B	1	"
Thiamine chloride	200	γ
Pantothenic acid	200	"
Riboflavin	200	"
Nicotinic acid	200	"
Pyridoxine	400	"
Pyridoxal	50	"
Folic acid	50	"
PABA	100	"
Biotin	1	"
Vitamin B_{12}	0.04	"
Tween 80	0.2	ml
pH 5.6-5.8 and dilute to	100	"

* 5 ml double strength medium used per tube. Addition is made to 5 ml of water or suitable aliquot of material under test.

* We are indebted to Dr. I. C. Gunsalus for this culture.

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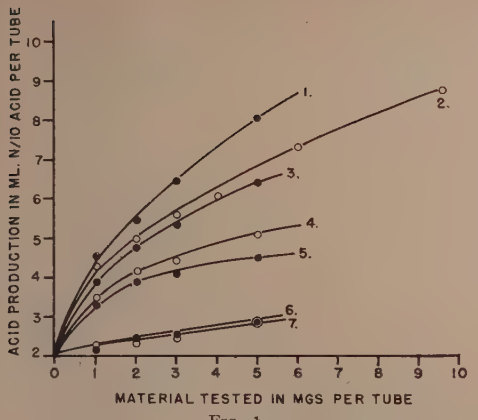

FIG. 1.

The response of *Lactobacillus bulgaricus* 09 to various proteins and protein digests. (1) Tryptic digest of vitamin-free casein. (2) Tryptic digest of vitamin-free casein treated twice with norit. (3) Undigested vitamin-free casein. (4) Tryptic digest of gelatin. (5) Tryptic digest of crude egg albumin. (6) Undigested gelatin. (7) Undigested crude egg albumin.

by dispersing 0.2 ml of a 24-hour culture from milk in 10 ml of sterile saline. One drop per assay tube was the routine inoculum used. Tests were incubated at 37°C for 48-72 hours. Relative growth was determined either by titration of the acid produced using 0.1 N NaOH with bromthymol blue as the indicator, or by measurement of turbidity in a photoelectric colorimeter.

The basal medium had the composition given in Table I. Although the organism does not appear to have a requirement for vitamin B_{12} this factor has been included in the basal medium in an attempt to increase the specificity of the response to "strepogenin" since Welch and Wilson have reported(12) that norit-treated tryptic digests of vitamin-free casein contain a factor capable of replacing vitamin B_{12} for *Lactobacillus leichmannii*.

In the assays that were carried out with *Lactobacillus casei* a medium essentially that of Landy and Dicken(13) with the asparagine level increased to 5 mg/tube was used.

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The tryptic digests of the various proteins described in Fig. 1 were prepared as follows: One gram of each protein was suspended in 10 ml 0.8% $NaHCO_3$. Twenty mg of trypsin (Difco 1:250) were added to each protein suspension and the mixtures incubated under benzene at 37°C for 48 hours. The undigested proteins were treated similarly prior to assay except that the trypsin was omitted. The results summarized in Table II were obtained on digests prepared with crystalline trypsin. In this instance one tenth the quantities employed in the earlier series were used.

The synthetic peptides that were studied were sterilized by autoclaving separately in water at 120°C for 10 minutes and assayed by aseptic addition to previously autoclaved media prepared in the usual manner.

Results and discussion. The response of *Lactobacillus bulgaricus* 09 to a variety of proteins or enzymatic digests is shown in Fig. 1.

Lactobacillus bulgaricus 09 responds almost as well to undigested casein as it does to a tryptic digest. Norit treatment did not appreciably reduce the "strepogenin" activity of a tryptic digest of casein. Gelatin and crude egg albumin have little growth-promoting activity for *Lactobacillus bulgaricus* 09. Tryptic digestion increased the "strepogenin" activity of these products.

The "strepogenin" activities of a variety of protein digests determined with the proposed assay with *Lactobacillus bulgaricus* 09 and with the *Lactobacillus casei* procedure are summarized in Table II. Agreement between the two methods of assay is reasonably good. Thus it would appear that under the conditions employed the two organisms respond to the same factor, or factors. The assays with *Lactobacillus bulgaricus* 09 were much more satisfactory, however, with respect to reproducibility and the absence of "irregular" tubes and "drift" at varying assay levels.

Asparagine and glutamine (sterile filtration, aseptic addition to previously autoclaved assay) were tested at levels up to 100 γ/tube. Neither compound stimulated *Lactobacillus bulgaricus* 09. Woolley reported(7) that glutamine stimulates *Lactobacillus casei* in

TABLE II.
"Strepogenin" Activities of Various Tryptic Digests.

Material studied	*Lactobacillus bulgaricus* 09 assay*	*Lactobacillus casei* assay*
Casein (Labco vitamin-free)	1.0	1.0
Twice norited casein (Labco vitamin-free)	0.53	0.55
Ribonuclease (crystalline)	0.67	—
Trypsin (crystalline)	1.2	1.3
Gelatin (Difco)	0.36	0.31
Protamine (purified)	0	—
β-Lactoglobulin (crystalline)	0.78	0.70

* Results are calculated in terms of trypsinized vitamin-free casein assigned arbitrarily an activity of 1.0.

the "strepogenin" assay at levels of about 3-30 γ/tube.

The following synthetic peptides have been examined at levels up to 4000 γ/tube: Glycylglycine, glycylglycylglycine, L-alanylglycylglycine, L-alanyl-L-alanine, L-leucylglycine, glycyl-L-leucine, glycylglycyl-L-leucylglycine, L-leucylglycylglycine, glycyl-L-glutamic acid, L-isoglutamine, glycylglycyl-L-glutamylglycine, α-L-glutamyl-L-glutamic acid, L-serylglycine, L-seryl-L-alanine, L-seryl-L-serine, L-serylglycyl-L-glutamic acid, L-seryl-L-alanyl-L-glutamic acid, glycyl-L-phenylalanine, L-glutamyl-L-phenylalanine, glycyl-L-tyrosine, L-tyrosylglycine, L-glutamyl-L-tyrosine, glycyl-L-glutamyl-L-tyrosine, glycyl-L-tryptophane, L-leucyl-L-tyrosine, glycyl-L-proline, glycylglycyl-L-proline, glycyl-L-prolyglycine, L-prolyl-L-glutamic acid, L-prolyl-L-tyrosine, glycyl-L-hydroxyproline, L-hydroxyprolylglycine, DL-methionylglycine, L-methionylglycylglycine, L-methionyl-L-methionine, L-methionyl-L-tyrosine.

At the levels tested none of the peptides showed activity. Woolley has reported(6,7) that DL-serylglycylglutamic acid has some activity in the *Lactobacillus casei* test over a range of about 200-3000 γ/tube. Certain other synthetic peptides have a lower order of "strepogenin" activity. The activity of serylglycylglutamic acid has been confirmed by Krehl and Fruton(14) using L-serylglycyl-L-glutamic acid. Thus it would appear that the *Lactobacillus bulgaricus* 09 assay is more specific than the *Lactobacillus casei* assay since in the present study such peptides as well as asparagine and glutamine had no growth-promoting properties.

A thorough study of recently identified growth factors and vitamins in the proposed assay for what appears to be "strepogenin" has not been made. Through the courtesy of Dr. G. Fraenkel a concentrate of vitamin B_T (15) which, like "strepogenin", is not well adsorbed on norit, has been tested and found to be inactive in the present assay. Most, if not all, of the uncharacterized bacterial growth factors and vitamins that have been described in the recent literature are adsorbed on norit or have not been found to be associated with purified proteins and consequently would not be expected to promote growth in the present assay.

Summary. A microbiological method for the determination of "strepogenin" activity is presented that depends upon the *essential* nature of the material for a strain of *Lactobacillus bulgaricus*. The method is believed to possess advantages over existing methods that depend upon the *stimulation* of the growth of *Lactobacillus casei*.

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Distribution of Radioactive Silver Colloids in Tissues of Rodents Following Injection by Various Routes.* (18017)

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During the last 4 years, colloidal manganese dioxide and colloidal gold containing the radioactive isotopes of the metals have been used in this laboratory in therapy of diseases of the reticulo-endothelial system(1). Colloidal radioactive gold has also been employed in a direct infiltration of tumor masses(2). Since Ag^{111}, a strong β-emitter with half-life of 7.6 days, meets the criteria essential for isotope therapy(3), it was deemed desirable to study the distribution of colloids prepared from this isotope. Voigt(4) used chemical analysis in his studies on colloidal silver localization. Gross microscopic localization was obtained by Shouse and Whipple(5) in their studies on the effects of intravenous injection of colloidal silver on the hematopoietic system in dogs.

Preparation of silver colloids. Due to the non-availability in the literature of methods for preparation of commercial silver colloids, 2 empirically devised procedures were used. Ag^{111} was obtained from neutron-bombarded palladium shipped from the Oak Ridge pile and separated by the method of Rouser and Hahn(6). The acidity of the HNO_3 solution of Ag^{111} was reduced by repeated alternate drying and solution in water and then made up to a convenient volume in a volumetric flask. One ml of a high dilution of the $Ag^{111}NO_3$ in the flask was counted over a period of 2 weeks on the thin mica end-window Geiger-Muller counter and followed the decay curve of Ag^{111} ($T/2 = 7.6$ days). A gelatin-protected silver colloid was prepared by thoroughly mixing 4 ml of the $Ag^{111}NO_3$ with 2 ml of liquid 8% gelatin.† The addition of 4 ml of N/40 NaOH resulted in a brownish appearance due to silver oxide formation. Ascorbic acid solution was added dropwise with thorough mixing to pH 7.4. The added ascorbic acid was considerably more than that calculated to effect reduction of the silver nitrate (containing 1 mg of carrier Ag per ml plus a minute amount of Ag^{111}). This preparation was diluted to 25 ml and allowed to stand overnight and on the next day (date of injection) 1 ml had a count of 310,000 per minute. A dextrin-protected colloid was prepared for the rat series. Separation and preparation of the 25 ml of $AgNO_3$ containing some Ag^{111} was carried out in the same way as with the gelatin-protected colloid. Ten ml of this solution was dried and dissolved in 5 ml of water and mixed thoroughly with 10 ml of a freshly prepared 2% dextrin solution. Next 12.5 ml of N/40 NaOH was added, followed by dropwise addition of ascorbic acid to a pH of 7.85. The dark brownish-red colloid was allowed to stand overnight and was filtered the next morning with loss of some metallic silver. One ml of the filtered colloidal preparation at this time possessed an activity of 1.72×10^6 counts per minute.

Experimental procedure. The gelatin-silver colloid was injected intraperitoneally into 5 white laboratory mice. One ml of the dextrin-silver colloid was injected into the caudal vein of 10 white laboratory rats, intramuscularly

* This work was carried out under contract with the Division of Biology and Medicine, United States Atomic Energy Commission.

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† Knox P-20 gelatin prepared from collagen was furnished through the courtesy of Dr. D. Tourtellotte of the Knox Gelatin Protein Products Co., Camden, N.J.

TABLE I.
Distribution of Ag^{111} Incorporated into a Gelatin-Protected Silver Colloid Injected Intraperitoneally in Mice.

	A		B		C		D		E	
Mouse										
Inj. sacrifice interval	22 hr		24 hr		30 hr		31 hr		32 hr	
	CPM	% total CPM inj.	CPM	% total CPM inj.	CPM	% total CPM inj.	CPM	% total CPM inj.	CPM	% total CPM inj.
Liver	25540	16.5	7813	6.3	7796	8.4	3865	4.2	11560	7.5
Spleen	1620	1.0	2188	1.8	1038	1.1	5580	6.0	2145	1.4
Heart	43	.0	43	.0					38	.0
	Total	Total	Sternum and 2 femurs		Sternum and 2 femurs		Sternum and 2 femurs		Total	Total
Bone	5350	3.5	255	0.2	108	0.1	197	0.2	4120	2.7
Skin (except of head)	647	0.4							1724	1.1
G. I. (tract and contents)	56500	36.5	13940	10.4	18878	20.1	28100	30.2	28700	18.5
Kidney	263	0.2	288	0.2					325	0.2
Muscle	15820	10.2							15600	10.1
Lungs	618	0.4	420	0.3	681	0.7	141	0.2	1060	0.7
Residual	3970	2.6							9167	5.9
CPM recovered	110471								74401	
% recovery		71.3								47.8

Mice A and E received each 0.5 ml of the gelatin-protected colloid with a count of 155,000; Mouse B received 0.4 ml with a count of 124,000; and Mice C and D received 0.3 ml with a count of 93,000. All counts are corrected to time of injection.

into the right hamstrings of a group of 3, and by cardiac puncture into a group of 2 without any observable toxic manifestations. Chemical analysis indicated that 1 ml of the dextrin-silver colloid contained only about 0.1 mg of silver.

The animals were sacrificed at varied intervals following injection as indicated in Tables I and II. The exsanguinated blood was collected in the beaker reserved for residual carcass and in 3 cases 1 ml was taken for counting. Organs, tissues, and extremities were dissected immediately, weighed and placed in beakers. Dry ashing was carried out in a muffle at from 625-650°C until complete. The ash was then dissolved in a minimum amount of concentrated HNO_3. In the case of organs known from preliminary experiments to concentrate only a small amount of silver and also those with little inorganic salt content in the ash, this HNO_3 solution was directly decanted into a seamless tin counting cup,‡ the bottom of which had been layered with concentrated NH_4OH to prevent corrosion of the cup by HNO_3. Decantations of water and ammonium hydroxide washings of the beakers were made into the counting cup. The NH_4OH was allowed to stand several hours in the beaker to dissolve silver precipitated by tissue chlorides. With organs such as the liver which concentrate large amounts of silver and also with bone, muscle, and residual carcass, in which the inorganic salt content after ashing is high, it was necessary to make decantations into a volumetric flask. The final solution in the flask should be somewhat acid to prevent precipitation of inorganic salts which occurs if the volume of NH_4OH washings renders the solution alkaline. After thorough shaking aliquots of 1 to 3 ml were taken and transferred to counting cups containing a thin layer of NH_4OH. After these samples were dried under an infrared lamp, their activities were determined by placing them under a standard shielded bell-type end-window Geiger-Muller counter.

Experiments. The results obtained with

‡ ¼-oz. size obtained from Buckeye Stamping Co., Columbus, Ohio.

TABLE II.
Distribution of Ag^{111} Incorporated into a Dextrin-Protected Silver Colloid Injected into Rats.

	Intravenous group										Intramuscular group			Cardiac puncture group	
Rat No.	IV-1	IV-2*	IV-3	IV-4	IV-5	IV-6	IV-7	IV-8	IV-9	IV-10	IM-1	IM-2	IM-3	CP-1	CP-2
Inj.-sacrifice interval, days	1 da.	5 da.	6 da.	8 da.	9 da.	12 da.	12 da.	12 da.	14 da.	14 da.	2 da.	6 da.	13 da.	5 da.	9 da.
Liver	1219.00	124.60	502.40	170.30	118.20	335.80	152.40	106.00	106.20	163.10	25.70	16.50	Lost	141.00	77.80
Spleen	46.61	10.26	9.95	6.94	10.40	Lost	7.26	12.32	3.81	5.12	4.50	2.35	5.76	10.72	2.02
Heart	.56	.24	.09	.15	.29	.44	.13	.28	.28	.04	.23	.15	.13	Lost	.27
Bone		RF .38	17.10T			39S	6.40T			.65S	.20S	.22S		31.40T	26.20T
Inguinal Lymph nodes	.14	.49†	.08	.08							.21				
Skin‡	52.20	8.59	52.60	44.80	42.40	31.80	25.20	14.25		19.20	36.30	23.25	13.00	59.40	41.60
G.I. tract and contents	192.70	19.53	33.70	26.70	45.90	63.70		25.10	23.90					64.92	45.35
Mesentery	5.42				1.00	3.85			2.05						
Kidney		4.45	1.79				1.18	.71		.54				3.09	4.85
Lungs						4.25			3.23		.96	.49		8.02	2.05
Right leg (site of i.m. inj.)											1529.00	1248.00	1089.00		
Left leg											4.20	4.70	.73		
Residual carcass	70.65	233.70	51.58	44.18	72.90			48.31			62.50	54.20	35.80		118.60
CPM recovered	1587.28	1339.80*	669.29	294.18	291.09			218.88§			1663.80	1349.86			360.08
% recovery	92.3	77.9	38.9	17.1	16.9			12.7			96.7	78.4			20.9

Rats received 1 ml of a dextrin-protected silver colloid calculated to contain 1.72×10^6 CPM on date of injection by the route indicated. Thousands of CPM for all tissues are corrected for decay to the date of injection (8/4/49) for comparison.

T—Total; S—Sternum; RF—Right Femur.

1 ml of blood in IV-2 gave 1360 CPM; in IV-6, 684 CPM; in IV-10, 160 CPM. Total recoverable muscle in IM-1 gave 50.60 thousand CPM; in IM-2, 41.34 thousand CPM. 90 mg of fecal pellets gave 1020 CPM in IV-4; 819 mg, 1100 CPM in IV-7; 300 mg, 850 CPM in IV-10.

* Rat IV-2—From results, it is probable that the injection for the most part was subcutaneous in the tail. (936.20 thousand CPM in tail.)

† Includes axillary nodes.

‡ Exclusive of head skin.

§ Includes 11.91 thousand CPM in the tail.

the intraperitoneal injections into mice are summarized in Table I. Excluding the intestinal tract through which elimination is taking place, the liver leads in content of silver, followed by muscle, bone, spleen, skin, and lungs. However, when activity is referred to counts per minute per unit of wet weight, the order of decreasing activity is found to be spleen, liver, bone marrow, lungs, muscle, and skin. The highest silver concentration is thus found in organs of the reticulo-endothelial system and resembles the distribution of colloidal iron following intravenous administration(7). In determining the bone marrow activity, it was assumed that the bone content of silver is primarily in the marrow(5) and that the bone marrow weight is 2-3% of the body weight(8). The feces were not collected, but complete recoveries of the silver were attempted from the carcasses of mice A and E. The lower percentage (47.8%) of silver activity recovered from Mouse E at 32 hours after injection as compared with recovery of 71.3% from Mouse A 22 hours after injection, together with the high content in the gastro-intestinal contents suggests elimination of ionic silver by that route. This elimination via the gastro-intestinal tract may be due: 1. to a large ionic silver content in the colloidal preparation§, 2. to possible *in vivo* conversion of colloidal silver into the ionic form, or 3. to elimination of colloidal silver. There is no known evidence for the elimination of more than trace amounts of silver in the urine.

The data obtained with the injection of the dextrin-silver colloid into rats is presented in Tables II and III. The most striking results were obtained with the 3 rats receiving the colloid intramuscularly in the right hamstrings. Almost 90% of the injected activity was recovered in the vicinity of the site of injection in one rat 6 days following injection and 63.3% of the injected activity was re-

TABLE III.

Counts Per Minute Per Milligram of Wet Weight Tissue in Rats Injected with Silver-Dextrin Colloid.

Group	Intravenous										Intramuscular			Cardiac puncture	
Rat	1	2	3	4	5	6	7	8	9	10	1	2	3	1	2
Liver	292.9	16.1	69.4	22.3	15.5	44.8	15.8	11.7	12.6	26.9	5.7	2.4	—	16.9	13.1
Spleen	350.5	31.1	35.3	16.3	28.1	—	11.7	28.9	10.1	22.8	19.9	12.7	11.6	27.7	6.2
Heart	1.7	0.4	0.1	0.2	0.3	0.7	0.1	0.4	0.4	—	0.4	0.3	0.2	—	0.5
Lungs						3.8			3.3		1.2			7.3	2.4
Bone Marrow			4.1											7.0	7.0
Mesentery	7.5				1.7	3.9			1.9						

7. Hahn, P. F., and Whipple, G. H., *Am. J. Med. Sci.*, 1936, v191, 24.

8. Maximow and Bloom, *Textbook of Histology*, 5th Edition, p. 85.

§ Conductivity measurements for ionic silver content were not carried out.

covered in another rat 13 days following injection. This high degree of localization suggests that radio-silver might be of considerable value in a direct infiltration of a tumor mass. Also, in the intramuscular group in the two animals for which data are available for both liver and spleen, the spleen possessed considerably greater activity per milligram of wet weight than the liver. Slower release to the circulation is possibly involved.

With the intravenous and cardiac puncture injections of the dextrin-silver colloid, again there is seen a diminishing percentage of injected activity recoverable with increasing injection-sacrifice intervals. Also, activity levels in the various organs show a general downward trend. Excretion of silver in the feces is again indicated by the high activity of the gastro-intestinal tract and also by the activity found in fecal pellets passed during the ether anesthesia preceding death. The activity of the stomach and its contents was measured in three animals and found to be insignificant. Assuming silver excretion in the bile, it seems probable that some is reabsorbed since the mesenteric content of silver per unit of wet weight is several times that of the heart or residual carcass. The silver content of the inguinal lymph nodes is slight. The blood level of silver is seen to decrease with time. The continued presence of silver in the blood is suggestive of translocation of silver deposited in the various tissues.

Reference to Table III indicates high but variable concentrations of silver in the liver and spleen. The silver concentrations of the liver and spleen for all the rats in the intravenous group except 1 and 6 and for the 2 in the cardiac puncture group were averaged. No. 1 was omitted due to the brevity of the injection-sacrifice interval and No. 6 since the spleen sample was lost. The average for liver was 22.0 cpm/mg of wet weight and for spleen was 21.8. This seems to indicate that, in spite of wide individual variations, the phagocytic propensities of the 2 organs per unit weight are approximately the same for colloidal silver if a large group is considered. Bone marrow, lungs, and mesentery followed in order of decreasing activity. It is of interest, however, that with the mice the activity of bone marrow followed quite closely that of spleen and liver; whereas, with the rats it was considerably lower. However, the colloidal preparation, species, and mode of injection were different.

Summary. 1. Intramuscular injection of a dextrin protected radio-silver colloid results in a high concentration of the injected material at the site of injection. 2. Intravenous injection of a dextrin-silver colloid into rats and intraperitoneal injection of a gelatin-silver colloid into mice result in deposition of the silver in highest concentration in the reticuloendothelial system. 3. Considerable silver was eliminated through the intestines and feces. The possible causes of such elimination under the conditions of this experiment are discussed. 4. A method of preparing tissues of small bulk for determining radio-silver activity with the Geiger-Muller counter is described.

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Experimental Pulmonary Edema. IV. Pulmonary Edema Accompanying Trauma to the Brain.* (18018)

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Boyd in his textbook notes that "Pulmonary edema is a very common condition" and ". . . occurs in a bewildering variety of conditions . . ."(1). One of these is skull fracture

* This study was supported by a grant from the Life Insurance Medical Research Fund.

1. Boyd, W., *The Pathology of Internal Diseases*, 4th Ed., Lea & Febiger, Phila., 1944, 213.

TABLE I.
Influence of Various Factors on Pulmonary Edema Resulting from Trauma to the Brain.

Exp. No.	Sex* M	Sex* F	Avg body wt, g	Lung edema % grp.	Lung edema Avg degree	Lung wt % body wt	Head injury	Treatment
1	5	5	210	0	0	.62 ± .09	0	
2	12	2	277	100	2.3+	1.04 ± .38	+	
3	8	0	260	100	2.6+	1.14 ± .25	+	Adrenalectomy
4	0	14	253	21	.3+	.76 ± .07	+	Pentobarbital
5	4	4	244	12	.1+	.62 ± .14	+	Etherized
6	10	0	267	30	.3+	.65 ± .25	+	Chloral hydrate
7	12	0	296	50	.6+	.59 ± .11	+	SKF-501
8	9	0	283	55	.5+	.68 ± .18	+	SY-2
9	10	0	202	50	1.2+	.85 ± .19	+	C-7337
10	0	12	191	75	1.7+	.78 ± .16	+	"

* Also indicates number of rats in each group.

Notes:

Exp. 3—Adrenalectomy 3 hr before.

4—Received 1 cc 5% sodium pentobarbital by stomach tube. All animals were comatose.

5—Deep ether anesthesia.

6—Given .5 g/kg chloral hydrate in 7.5% sol by stomach tube. Produced deep narcosis.

7—SKF-501 is an adrenergic blocking agent, N-(9-fluorenyl)-N-ethyl-β-chlorethylamine HCl, obtained from Smith, Kline & French. 2 mg/kg in 1.5 cc saline were given 30 min. before the test.

8—SY-2 is an adrenergic blocking agent, N-dimethylamino-2-propyl-1-thiodiphenylamine HCl, obtained from Parke, Davis Co. A dose of 2.5 mg/kg in 2 cc was given i.v. 30 min. before the test.

9—C-7337 is an imidazoline derivative: 2[N-p'-tolyl-N-(M'-hydroxyphenyl)-aminomethyl] imidazoline HCl with adrenergic blocking properties which was obtained from Doctor F. F. Yonkman of Ciba. The dose used was 50 mg/kg by stomach tube 2 hrs before the test.

10—Compound used in Exp. 10 was administered intraperitoneally 30 min. beforehand.

and there are numerous reports of lung edema following head injuries(2-9). Pulmonary edema due to epinephrine injections(10), toxic doses of ammonium salts(11) and hypoglycemia(12) is in all cases prevented by sympathetic nerve blocking agents. The influence of these and other factors upon the lung edema due to skull fracture in the rat has been determined.

Experimental. Albino rats at rest had their heads between 2 laterally placed wooden blocks one of which was fixed. A sudden static force sufficient to produce a fatal head injury was applied to the other block. This caused no pain, unconsciousness being instantaneous and the animals were dead in a matter of seconds or several minutes at most during which their were clonic convulsive movements. Almost without exception rats killed in this manner showed some degree of pulmonary edema. It was graded by inspection (0, 1± - 4±) and a quantitative measure made by recording the weight of the lungs in relation to body weight. It is true that when quantitative estimations of the degree of pulmonary edema present at autopsy are desired rapid exsanguination gives the most satisfactory preparation of the lungs (13). This was used in earlier experiments and in the present animals insofar as there was any blood flow.

2. Moutier, F., *Presse Med.*, 1918, v12, 108.

3. Antonini, A., and Biancalani, A., *Arch. anthropol. criminale*, 1927, v47, 747.

4. Astuni, A., *Minerva Med.*, 1934, v1, 380.

5. Benassi, G., *Paris Med.*, 1937, v1, 525.

6. Weisman, S. J., *Surgery*, 1939, v6, 722.

7. Schlesinger, B. J., *Nerv. and Ment. Dis.*, 1945, v102, 247.

8. Luisada, A., *Medicine*, 1940, v19, 475.

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11. MacKay, E. M., and Pecka, E. F., Jr., PROC. SOC. EXP. BIOL. AND MED., 1950, in press.

12. Luisada, A., *Arch. Exp. Path.*, 1928, v132, 313.

13. Durlacher, S. H., Baufield, W. G., and Bergner, A. D., *Fed. Proc.*, 1950, v9, 35.

Results. The experiments are summarized in Table I. The pulmonary edema produced by brain trauma (Exp. 2) is very striking. The absence of the adrenals (Exp. 3) failed to influence it.

Narcosis produced by various agents (Exp. 4, 5, 6) protected against the development of pulmonary edema. Adrenergic blocking agents (Exp. 7, 8, 9, 10) were also effective in varying degree.

Discussion. As has been noted above, the occurrence of other types of experimental pulmonary edema is also inhibited by adrenergic blocking agents(10,11,12). It has likewise been reported that narcosis prevents experimental pulmonary edema due to epinephrine in some species(14) inhibits ammonium pulmonary edema(11) and confers the best protective effect against the lung edema due to massive intracarotid infusions of saline(15). There is every indication that these various types of pulmonary edema are fundamentally similar in their origin and result from nerve stimuli of central origin, *i.e.*, they are all types of neurogenic pulmonary edema. The mechanisms through which these stimuli produce the edema is less clear. Visscher's group has stressed(16) the acute bradycardia and rise in pulmonary venous pressure associated with pulmonary edema. They find(16,17) that these changes precede the pulmonary edema due to increased intracranial pressure. The latter is essentially the same as the pulmonary edema which is produced more rapidly by traumatic injury of the brain. Campbell and Visscher(17) report that bilateral cervical vagotomy performed immediately beforehand in guinea pigs prevents or reduces the degree of lung edema due to increased intracranial pressure. We have examined the effect of this in brain trauma in guinea pigs and it is true that the lung edema is less severe. The most noticeable effect is the failure of a bradycardia to ensue. A tachycardia may even result. However bilateral cervical vagotomy alone eventually leads to the development of pulmonary edema and the problem appears to be not so simple as a question of efferent sympathetic stimuli from the brain which may be reduced by narcosis or adrenergic blockade and prevented by interrupting their flow through section of the vagi.

Summary. Experimental pulmonary edema occurs in the rat in a matter of seconds as a result of a blow to the calvaria causing fatal trauma to the brain. This edema is prevented or reduced in degree by agents which produce a deep narcosis and by adrenergic blocking compounds. The mechanism of its production is discussed in relation to other types of experimental pulmonary edema.

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Prolytic Changes in Chicken Erythrocytes Exposed to *Micrococcus aureus* Toxins.* (18019)

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Bernheimer(1,2) exposed human erythrocytes to the toxin of *Clostridium septicum* for

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1. Bernheimer, A. W., *J. Gen. Physiol.*, 1946, v30, 337.

20 minutes or more and then removed the toxin. He postulated that the toxin, possibly acting catalytically, combined with the erythrocyte resulting in an altered cell which swelled and then hemolyzed. These observations confirmed those of Menk(3) and conform to the hypothesis proposed by Wilbrandt (4) who suggested that some lysins increase the cation permeability of the erythrocyte which leads to osmotic hemolysis (*cf.* Davson, 5). In the present investigation chicken erythrocytes were exposed for a short period of time to a dilute toxin, which at higher concentrations had been shown to be hemolytic (6), and the toxin was then removed by washing. Such experiments should give some information concerning the interaction of toxin and cell. In addition, by using a concentration of toxin which produced hemolysis slowly, a long period of time was available for studying prolytic changes in the cells.

Materials and Methods. Four stock suspensions were made as follows: Equal volumes of chicken erythrocytes (obtained from heparinized blood) and Ringer Locke were placed in 2 vials and equal volumes of erythrocytes and a 1:100 dilution in Ringer Locke of *Micrococcus aureus* toxin (15,000 dermal necrotic doses/cc) were placed, in 2 other vials. These 4 vials were immediately put into a water bath at 37°C and kept at this temperature except for brief intervals required for centrifugation and other manipulations described below. After 10 minutes the contents of one control vial (A) and one experimental vial (B) were centrifuged, the supernatant fluids discarded and the cells resuspended in 10 cc of Ringer Locke. This gave a control (A) and an experimental (B) in which the toxin had been removed and the cells resuspended in Ringer Locke and a control (C) and an experimental (D) which had not been centrifuged. After various periods of incubation at 37°C the following observations were made: (1) A and B were centrifuged and the amount of hemoglobin in the supernatant fluid was measured by determining the percent transmission at 545 mμ using a Coleman Universal Spectrophotometer. This series of observations indicated the progress of hemolysis in stock suspensions A and B. (2) The rate of swelling was measured using the photoelectric apparatus previously described(7). An aliquot (0.09-0.13 cc) of each stock suspension (the centrifugate of A and B; C, D) was added to 10 cc of a solution of 0.3 M glycerol in Ringer Locke in the chamber of the apparatus. A Kipp and Zonen galvanometer was used and records were obtained on 12 cm bromide paper. These observations indicated the change in osmotic behavior that had occurred in the cells. (3) The glycerol—Ringer Locke —cell suspensions were removed from the photoelectric apparatus, centrifuged and the amount of hemolysis determined spectrophotometrically. These measurements indicated changes in "fragility"‡ of the cells. (4) Fragility measurements were made by adding aliquots of suspensions A-D to a series of tubes containing dilutions of NaCl from 1-0%. Following equilibration at 37°C the contents of these tubes were placed in the permeability apparatus and a record obtained. (5) Rates of hemolysis in ethylene glycol and glycerol (0.3M) were measured using the photoelectric apparatus. (6) Equal volumes of the stock suspensions (A-D) and 0.3 M glycerol in Ringer Locke were mixed and left at 37°C for at least 10 minutes. The rate of shrinking was recorded by adding an aliquot of these new suspensions (A′-D′) to 10 cc of Ringer Locke in the permeability apparatus. This technic, described by Wil-

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3. Menk, W., *Zent. f. Bakt.*, 1931, v123, 55.

4. Wilbrandt, W., *Pfluger's Arch.*, 1941, v245, 22.

5. Davson, Hugh, *Biochem. J.*, 1936, v30, 391.

6. Hunter, F. R., Rawley, June, Bullock, Jane A., and Larsh, Howard W., *Science*, 1950, v112, 206.

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‡ The quotation marks are used to indicate that a different type of measurement is involved in obtaining these values. In measuring fragility cells hemolyze as a consequence of the hypotonicity of a salt solution. In measuring "fragility" the cells hemolyze as a consequence of shrinking and swelling to their initial volume(8).

TABLE I.

A typical experiment indicating the progressive hemolysis which occurred in washed chicken erythrocytes which had been exposed to the toxin of *M. aureus* for 10 min. at 37°C. Data were obtained spectrophotometrically and compared with a standard hemolysis curve. The values indicate the amount of hemolysis which had occurred since the preceding measurement while standing at 37°C.

	% hemolysis	
Time in hrs	Control (A)	Experimental (B)
½	1	< 1
5	< 1	< 1
11	< 1	1
27½	4	9
53	11	>25

TABLE II.

A typical experiment indicating the progressive increase in "fragility" (measured spectrophotometrically as hemolysis occurring in a solution of 0.3 M glycerol in R.L.) of chicken erythrocytes exposed to the toxin of *M. aureus* (one pair washed (A,B), the other pair unwashed (C,D)).

	% hemolysis*			
Time in hrs	A	B	C	D
½	1.6	1.6	1.3	0.8
14	2.5	3.8	2.0	7.8
23½	2.5	2.5	3.5	12.2
41	3.5	3.5	3.8	29.4
61	3.8	11.8	13.1	40.5

* These values include the hemolysis which occurred in the stock suspension prior to shrinking and swelling.

brandt(9), gives a measure of permeability to supplement the swelling data. (7) These Ringer Locke suspensions were centrifuged following (6) above and the percent of hemolysis was determined spectrophotometrically. This gives additional information concerning the fragility of the cells.

Following these observations, 10 cc of Ringer Locke were added to A and B and they remained at 37° until some later time when the above observations were repeated. Bacteriologically sterile technics were used except for the few minutes required to make the actual observations. Sterility tests were made as previously described(10).

Results. Table I shows the progressive hemolysis in A and B. As one might expect, the control cells hemolyzed slowly over a period of many hours while the cells which had been exposed briefly to the dilute toxin hemolyzed a little more rapidly. Table II indicates what percent of the cells hemolyzed when allowed to shrink and swell in a hypertonic solution of a penetrating molecule in a balanced salt solution. As indicated by D, the toxin in this dilution affects the cells slowly, one of the prolytic changes being a progressive increase in "fragility" beginning about 12-13 hours after exposure to the toxin. Cells which have been exposed to the toxin for only 10 minutes (B) show a similar progressive increase in "fragility" but in general this seems to occur more slowly. A comparison between A and C shows no consistent effect of washing and centrifuging the cells on "fragility," although in most of the experiments the cells in A appeared to be more "fragile" (*cf.* Davson and Danielli,11).

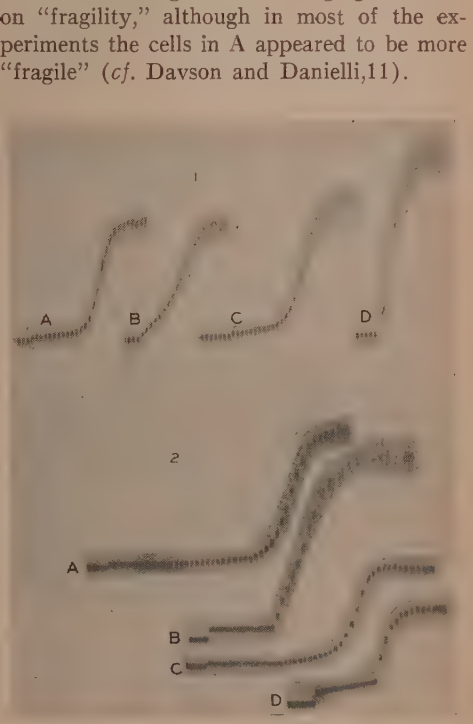

FIG. 1.

Effect of the toxins of *M. aureus* on the rate of hemolysis of chicken erythrocytes in 0.3 M ethylene glycol (1) and in 0.3 M glycerol (2). Time intervals: 1—sec; 2—first 15 sec. continuously, every 15 sec. A—washed control; B—washed toxin; C—unwashed control; D—unwashed toxin.

9. Wilbrandt, W., *Pfluger's Arch.*, 1941, v245, 1.

10. Hunter, F. R., Marker, Muriel J., Bullock, Jane A., Rawley, June, and Larsh, Howard W., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 606.

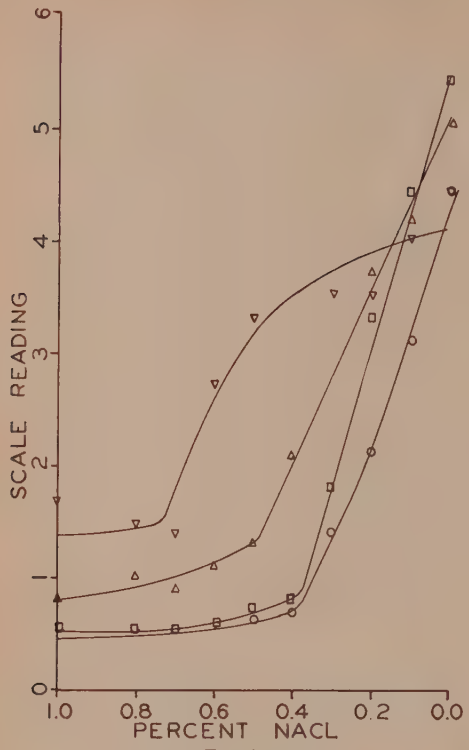

FIG. 2.

Effect of the toxins of *M. aureus* on the fragility of chicken erythrocytes. ○—washed control; (A); △—washed toxin (B); □—unwashed control (C); inverted triangle—unwashed toxin (D).

Fig. 1 shows hemolysis in ethylene glycol and glycerol. The time for hemolysis is essentially the same with the washed and unwashed control cells (there is some indication in most of the experiments that the washing altered A cells slightly), while the cells which had been exposed briefly to the toxin (B) hemolyzed more rapidly and those cells still in the presence of the toxin (D) hemolyzed most rapidly. The same sequence was observed when fragility was measured (Fig. 2). The cells in D were most fragile, those in B less fragile and those in A and C least fragile. Fig. 3 shows the swelling of cells in 0.3 M glycerol in Ringer Locke and the shrinking of cells which had been equilibrated in this solution and then placed in Ringer Locke. Spectrophotometric measurements which were made after the shrinking curves had been obtained gave respectively the following values for per cent hemolysis: A′—55%; B′—58%; C′—34%; D′—>> 60%. These curves actually give some indication of the fragility of the cells, for as they swell, some reach their hemolytic volume. This is indicated at the top of the curve just before shrinking begins. The hemolysis values for these solutions given above are an index of fragility. In the case of the cells in D, they were so fragile (Fig. 2) that no shrinking curve could be obtained for most of the cells hemolyzed during their initial swelling.

Some interesting comparisons can be made between the data obtained from cells exposed to the toxins for 40-45 hours that have been discussed above and cells exposed for 5-7 hours. In the latter case the fragility, swelling and shrinking curves obtained with A, B, C and D are all essentially identical. Also, the shrinking curves show less hemolysis just prior to shrinking. Spectrophotometer read-

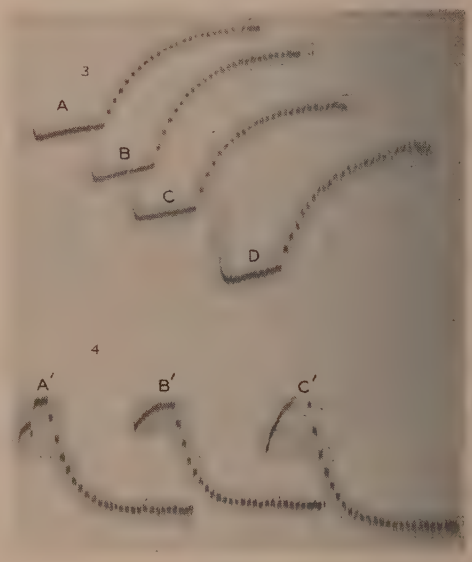

FIG. 3.

Effect of the toxins of *M. aureus* on the rate of swelling in 0.3 M glycerol in Ringer Locke (3) and the rate of shrinking in Ringer Locke following equilibration in 0.3 M glycerol in Ringer Locke (4). A,A′ washed control; B,B′—washed toxin; C,C′—unwashed control; D—unwashed toxin.

11. Davson, Hugh, and Danielli, J. F., *Biochem. J.*, 1938, v32, 991.

ings showed the following per cents of hemolysis for swelling and shrinking: A—8%; B—4%; C—7%; D—10%; A′—7%; B′—4%; C′—6%; D′—6%. The second series of values are another indication of little if any fragility change while the first series shows little or no increase in "fragility." After this length of time, then, the toxin had had little effect on the cells.

Discussion. The prolytic changes observed in these experiments are an increase in the fragility (and "fragility") of the cells and a decrease in the time for hemolysis in solutions of lipid insoluble non-electrolytes. The rates of swelling and of shrinking, however, are not altered to any appreciable extent. This suggests that there is little if any change in permeability, the difference in rates of hemolysis being a consequence of the change in fragility of the cells. It is of interest to note that similar observations have been made on chicken erythrocytes exposed to the toxins of *B. cereus*(12) and *Cl. perfringens*(13).

A comparison of the washed and unwashed toxin-treated cells suggests that using toxin dilutions which are weakly hemolytic, some irreversible change in the membrane occurs very rapidly (less than 10 minutes). Since a number of workers(14-17) have suggested various mechanisms including adsorption, fixation or complex formation between lytic agents and erythrocyte membranes, it would seem reasonable to suggest that some toxin stays with the cells while that which is uncombined is removed by the washing. Since the unwashed experimental cells changed more rapidly than those which had been washed it can be assumed that a small, fixed quantity of toxin will alter the cells gradually, but if a "reserve" of toxin molecules is present, the change in the cells will take place more rapidly. This conclusion is consistent with observations such as those of Ponder(18,19).

Although the preceding studies using saponin(20) and toxins of *Str. β-hemolyticus* (21) did not involve as complete an analysis as in the present experiments, there is reason to believe that the prolytic action of these two substances does differ from the action of the toxins of *M. aureus*. This is not surprising for although Ponder and McLachlan(22) reported a similarity in the kinetics of hemolysis by saponin and toxins, Bernheimer(1) has shown that this is not the case.

Conclusions. 1. Chicken erythrocytes exposed to a dilute toxin obtained from *Micrococcus aureus* progressively become more fragile prior to hemolysis. 2. These cells hemolyze more rapidly in isosmotic solutions of ethylene glycol and glycerol. 3. Swelling and shrinking measurements show that the toxin has little or no effect on the permeability of these cells to glycerol. 4. The fact that more of the toxin-treated cells hemolyze following shrinking and swelling in 0.3 M glycerol in Ringer Locke than do controls suggests that the toxin may weaken the membrane in some way. 5. A comparison between erythrocytes exposed to the toxin for 10 minutes and then washed and erythrocytes continuously in the presence of the toxin indicates that the toxin effects an immediate and irreversible change. 6. Even though diffusible toxin is removed by washing, some toxin apparently remains and gradually the cells hemolyze. This process occurs more rapidly if the diffusible toxin is not removed.

12. Unpublished results.
13. Unpublished results.
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Glutathione Potentiation of Cortisone-induced Glycosuria in the Rat.* (18020)

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Glutathione, which was first shown to protect against alloxan diabetes(1,2), may play a similar protective role in other types of diabetes(3). Since the glutathione content of the blood and tissues is decreased by injecting growth hormone(4), adrenocorticotrophic hormone(5), and cortisone(6), and since it has been claimed that gluthathione, when injected into human subjects with "ACTH-induced diabetes," caused a transitory decrease in glycosuria(7), we have studied the effect of glutathione administration on cortisone diabetes in the rat(8,6).

Methods. Normal and subdiabetic Sprague-Dawley rats were used in this study. The latter were prepared by starving 100 g rats for 48 hours and injecting with alloxan (15 mg/kg). Those rats which showed a decreased glucose tolerance but normal blood sugar values (subdiabetes) were selected and allowed to reach a weight of 300 g. Rats weighing 300 g were placed in metabolism cages and a high carbohydrate liquid diet† was fed by stomach tube in the morning and early evening. Although some of the rats developed an initial glycosuria, all of the normal rats became aglycosuric within a few weeks and the subdiabetic rats became aglycosuric within one month. Aqueous suspensions of cortisone acetate (Merck) containing 1.5% benzyl alcohol were injected subcutaneously immediately preceding each feeding. The dose totaled 4 mg per day for the subdiabetic rats and 10 mg per day for the normal rats. Urines were collected daily under toluene and the glucose excretion was determined by the caramelization method of Somogyi(11) adapted to the Klett Colorimeter.

Solutions of glutathione 0.5 M, cysteine (free base) 0.5 M, ascorbic acid 0.5 M and D,L-Alanine 1.0 M were neutralized to pH 7.4 and injected intraperitoneally in doses of 0.5 cc/100 g of rat 10 minutes prior to each feeding. When cortisone was also injected it was given immediately preceding the feeding.

Results. The effect of glutathione injection (5.0 mM/kg/day) on the sugar excretion of normal rats is shown in Table I. Although glutathione induced glycosuria in the normal rat, the amount of sugar excreted following successive injections of glutathione decreased progressively as the length of time on the high carbohydrate diet increased. After two months it was insignificant. Cysteine appeared to be less effective than glutathione for equimolar doses of the former, when injected on the 25th day, induced less glycosuria than did

* This investigation which was supported by a research grant from the National Institutes of Health, Public Health Service, was presented at the American Association of Anatomists' meeting, New Orleans, April 6, 1950.

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† The high carbohydrate liquid diet of Reinecke *et al.*(9) as modified by Ingle(10) was diluted with an additional 25% water and administered in increasing amounts over a 5-day period. Thirty-two cc/day were fed after the fifth day and the total caloric intake remained the same(10). This dilution of the diet facilitates the passage of the food through a No. 8 French catheter.

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TABLE I.

Effect of Glutathione on the Urinary Excretion of Glucose in Normal Rats.

Seven normal rats (Nos. 22-30) were forced-fed a high carbohydrate diet. These same animals were injected at various times with the substances indicated in doses of 5.0 millimoles per kilogram per day except DL-alanine which was injected in doses of 10.0 mM/kg per day. The average 24-hr glucose excretions are recorded.

			g of urinary glucose/24 hr			
			Pre-inj. level		Increase due to inj.	
Rat No.	Days on high carbohydrate diet	Substance inj.	Avg	Range	Avg	Range
22-30	18-21	Glutathione	.03	.00-.05	.66	.06-1.58
,,	32	,,	.03	.00-.08	.37	.02-1.31
,,	45	,,	.02*	.00-.04	.11*	.00- .27
,,	63	,,	.01†	.00-.03	.03†	.00- .11
14-17	47-54	,,	.01‡	.00-.03	.06‡	.01- .13
22-30	25	Cysteine	.04	.00-.07	.20	.00- .58
,,	37	Ascorbic A.	.03	.00-.06	.09	.05- .17
,,	29	Alanine	.02	.00-.07	.10	.00- .19

* Average of only 6 rats.

† Average of only 4 rats.

‡ Represents the average of 6 determinations on 3 new rats which received their first glutathione injection on day 47 and 2nd injection on day 54.

glutathione given on the 32nd day.

One normal rat (No. 18), was injected with cortisone (10 mg/day) beginning on the 33rd day of the high carbohydrate diet; glycosuria appeared on the fourth day, reached a maximum of 1.9 g/day on the 7th day and thereafter stabilized at a value of 0.3 to 0.5 g/day. Glutathione, injected on 3 separate days induced a ten-fold increase in glycosuria (up to 5.2 g/day). (Table II). In 4 additional normal rats injected with cortisone (10 mg/day), glutathione increased the glycosuria from 2 to 4½ times. In 2 cortisone diabetic rats (10 mg/day) in which the glycosuria had leveled off at about 2 to 3 g/day, glutathione injection caused death within 1 to 2 hours.

Smaller doses of cortisone induced glycosuria in subdiabetic rats. One such rat (No. G) which received cortisone (4 mg/day) beginning on the 57th day developed glycosuria after 4 days; this reached a maximum of 0.9 g after 6 days, and leveled off at about 0.5 g/day thereafter. At various times indicated in Table II this same rat was injected with glutathione, cysteine, ascorbic acid and alanine and the effects on sugar excretion were determined. Four sub-diabetic rats, which did not receive cortisone showed insignificant responses to cysteine and glutathione.

Discussion. In normal rats fed a high carbohydrate diet the injection of glutathione appears to bring out latent abnormalities in tolerance to carbohydrate which ultimately disappear after 6 to 8 weeks of the diet. The decreased effectiveness of successive doses of glutathione is not due to the development of a tolerance to injected glutathione because when the first glutathione injection is delayed until the 47th day of feeding, the glycosuria was nevertheless insignificant. The increased glycosuria which follows glutathione injection into cortisone-diabetic rats, occurs at a time when the control rats showed insignificant responses to glutathione. An increased excretion of cysteine or glutathione following sulfhydryl injection would not be measured as glucose since these compounds will not caramelize with sodium carbonate. Direct conversion of the injected glutathione to glucose could account for only a small fraction of the glucose excreted. Since preliminary studies indicated that the blood sugar of cortisone-diabetic rats is markedly increased following glutathione injection, the glycosuria is not simply the result of a decreased renal threshold.

Insulin is inactivated by cysteine and glutathione *in vitro*(12) and presumably also *in*

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TABLE II.

Effect of Glutathione on Urinary Glucose Excretion in Cortisone-Diabetic Rats.

Rats forced-fed a high carbohydrate diet. Test substances were injected in doses of 5 millimoles per kilo (except DL-alanine which was injected in doses of 10 mM/kg) on the days indicated.

Condition of rat	No. of rats	Dose cortisone		Days on high carbohydrate diet	Test substance	g of urinary glucose/24 hr			
		mg/day	days			Pre-inj. level		Increase due to inj.	
						Avg*	Range	Avg*	Range
Normal	3	0	0	47-54	Glutathione	.01	.00- .03	.06 (6)	.01- .13
"	1 (No. 18)	10	11, 14, 21	44, 47, 54	"	.38	.30- .48	3.75 (3)	1.84-5.17
"	4	10	10-23	18-31	"	.51	.40- .73	1.39 (4)	1.17-1.59
Sub-diabetic	1 (No. 6)	4	10, 22, 37, 51	67, 79, 94, 108	"	.55	.34- .71	1.80 (4)	1.45-2.44
"	" "	4	16, 32	73, 89	Cysteine	.82	.61-1.03	1.15 (2)	.50-1.80
"	" "	4	19, 44	76, 101	Ascorbic A.	.42	.20- .64	.61 (2)	.51- .72
"	" "	4	28	85	Alanine	.77		.35 (1)	—
"	4	0	0	89-116	Glutathione, cysteine, or ascorbic a.	.03	.00- .06	.05 (16)	.00- .20

* No. of determinations are given in parenthesis.

vivo(13). If the increased glycosuria resulting from sulfhydryl administration were the result of *in vivo* inactivation of insulin, one would expect the glycosuria following cysteine injection to be greater than that following glutathione, since cysteine is a more potent inactivator of insulin than is glutathione(12). This does not seem to be the case. Although the greatest sugar excretion was observed when glutathione was injected into cortisone-diabetic rats, it cannot be argued that these diabetic rats should show more glycosuria as a consequence of insulin inactivation than do normal rats, because Ingle has shown that cortisone-diabetic rats are highly resistant to insulin(14). Since glutathione can reduce the disulfide groups of protein to sulfhydryl, it may produce glycosuria through its effect on some disulfide dependent enzyme. Insulin, which requires disulfide groups for activity (15), may have an opposite effect.

Since cortisone injection results in an increased ketosteroid excretion(16) part of the injected steroid is destroyed by oxidation of the side chain. Reducing agents such as cysteine and glutathione, by protecting cortisone from destruction, may thereby potentiate its diabetogenic effect. Cysteine and probably glutathione have also been found to potentiate the carbohydrate storage (liver glycogen) effects of cortisone(17). Since glutathione also induced glycosuria in normal animals during the early period of high carbohydrate feeding, it may have an effect on endogenous steroids.

The increased glycosuria found in "cortisone-diabetic" rats treated with glutathione is contrary to the observations reported by

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Conn(7). He found that when glutathione was injected into man with "ACTH diabetes" there was a transitory decrease in glycosuria lasting 1 to 2 hours. Studies with ACTH are now in progress.

These results emphasize that although glutathione protects the beta cell against physiologically occurring toxic compounds(3) it may also have diabetogenic effects due to its action on steroids, enzyme systems, etc. They furthermore suggest the possibility that glutathione might potentiate the therapeutic effect of cortisone in the treatment of rheumatic disease.

Summary. Glutathione, when injected into normal animals which had been force-fed a high carbohydrate diet, induced glycosuria. The amount of glycosuria decreased with increasing adaptation to the high carbohydrate diet and after feeding the diet for 2 months, glutathione produced insignificant effects. By contrast the injection of glutathione into cortisone-diabetic rats caused a marked (in some cases up to 10 fold) increase in glycosuria which has been observed even after 3 months of the high carbohydrate diet. Cysteine and ascorbic acid were also studied. Glutathione potentiates the diabetogenic effect of cortisone. The mechanism of this potentiation has been discussed and its possible significance in the treatment of rheumatic disease has been suggested.

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Chemoprophylactic Effectiveness of Aureomycin and Terramycin in Murine Bartonellosis.* (18021)

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The bartonellae resemble the rickettsiae in many of their characteristics. Previous work in this laboratory (unpublished) has shown, however, that murine bartonellosis is not prevented or made less severe by para-aminobenzoic acid or penicillin, both of which are highly effective against murine typhus infection in mice(1,2). We have also obtained negative chemoprophylactic results in murine bartonellosis with sulfanilamide, sulfathiazole, sulfapyridine, folic acid, atabrine, and several other antimalarial drugs. The purpose of this paper is to report the chemoprophylactic effectiveness of aureomycin and terramycin in this disease.

Material and methods. Two strains of white rats were used, one of which was bartonella-free while the other, like most laboratory strains, was a so-called "carrier" strain, latently infected with *Haemobartonella muris*. It is well known(3) that rats rarely develop clinical or hematological evidence of bartonellosis unless splenectomy is carried out. When bartonella-free rats are splenectomized and then injected with the organism, they become severely ill in a few days as a result of extensive parasitization and destruction of erythrocytes, and the mortality is usually 100%. When ordinary carrier rats are splenectomized, they also become severely ill, and the mortality usually ranges from 50 to 75%.

In one experiment, 19 bartonella-free rats (Sprague-Dawley strain) were splenectomized, and injected 3 weeks later with 1 cc of rat blood containing *H. muris* of high virulence. Twenty-four hours after injection, 5 of these rats were started on aureomycin, 4 on terramycin and 5 on chloramphenicol, the remaining 5 serving as controls. The aureomy-

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TABLE I (Exp. 1).
Chemoprophylactic Effect of Aureomycin, Terramycin, and Chloramphenicol on Splenectomized Bartonella-free Rats Injected with *H. muris*.

Rat No.	Dosage	Illness after inj. (hr)	Lowest hemoglobin level (mg)	Highest Bartonella count (per 100 r.b.c.)	Hemoglobinuria	Outcome
1	Control	72	10	237	+	died 5th day
2		72	6	127	+	" " "
3		72	10.5	54	+	" " "
4		72	7	99	+	" " "
5		72	6	246	+	" 6th "
6	Chloramphenicol	96	7.5	289	+	" 7th "
7	18 mg twice daily,	96	9	143	—	*S
8	9 days	96	6	241	+	died 7th day
9		72	6	102	+	" 5th "
10		72	4	315	+	" 7th "
11	Aureomycin	0	16	0	—	*S
12	10 mg twice daily,	0	15	0	—	"
13	9 days	0	12.5	<1	—	"
14		20 days	16.5	<1	—	died 20th day†
		21 "	14.5	<1	—	" 21st "
16	Terramycin	0	16.5	0	—	*S
17	18 mg twice daily,	0	17	1	—	"
18	9 days	0	15	0	—	"
19		0	16.5	<1	—	"

* S Survived.
† Cause of death not obvious. No anemia and no bartonellae in blood at time of death.

cin was dissolved in distilled water and injected subcutaneously in the dosages indicated in Table I. The terramycin and the chloramphenicol were relatively insoluble in water and were given partly in aqueous suspension by the subcutaneous route.

In a second experiment, 30 carrier rats of the St. Louis University strain were splenectomized. Twenty-four hours later, 10 of these rats were started on aureomycin, 10 were started on terramycin, and 10 were left as controls. The drugs were given subcutaneously, as described above, and in the dosages shown in Table II.

Criteria for the effectiveness of the drugs were: 1. presence or absence of clinical illness, 2. daily direct hemoglobin determinations (Dare) for 10 days, 3. daily bartonella counts in giemsa stained blood films for 10 days, 4. presence or absence of hemaglobinuria (obvious by inspection) and 5. mortality. Bartonella counts were recorded as organisms per 100 erythrocytes, based on observation of 400 erythrocytes.

Parenteral aureomycin was obtained from the Lederle Laboratories, and terramycin from the Charles Pfizer Co.

Results. The results of these experiments are shown in Tables I and II. In Exp. 1, (Table I) all 5 control rats and 4 of the 5 rats receiving suspensions of chloramphenicol, developed high bartonella counts, severe anemia and hematuria, and died 5 to 7 days after injection with *H. muris*. There was evidence of a slight prolongation of the incubation period in the rats receiving chloramphenicol, and the fact that one of these rats survived is possibly significant, since 100% mortality is the rule in this type of experiment. None of the rats treated with aureomycin or terramycin showed clinical evidence of anemia or hematuria. Several of these animals showed low parasite counts for a day or two, but definite anemia did not develop in any during an observation period of 30 days. (Two rats from the group receiving aureomycin died on the 20th and 21st days after injection of bartonellae. The cause of death was not clear, but these rats did not show anemia or bartonellae in the blood stream.

In Exp. 2 (Table II) all 10 control animals developed severe clinical illness, severe anemia, and high bartonella counts. The mortality was 50%, which is about average

TABLE II (Exp. 2).
Chemoprophylactic Effect of Aureomycin and Terramycin on Splenectomized Carrier Rats.

Rat No.	Dosage	Illness after splenectomy, hr	Lowest hemoglobin level (mg)	Highest Bartonella count (per 100 r.b.c.)	Hemoglobinuria	Outcome
1	Control	144	7.5	99	—	*S
2		120	5	108	—	”
3		120	3	216	—	died 9th day
4		144	5	122	—	*S
5		120	5	240	—	”
6		120	5	220	+	died 8th day
7		144	4	266	+	” 7th ”
8		120	3	276	—	*S
9		120	6	211	—	died 6th day
10		168	6.5	363	—	” 8th ”
11	Aureomycin	None	11	0	—	*S
12	15 mg twice daily,	”	13	0	—	”
13	8 days	”	16	0	—	”
14		”	14	0	—	”
15		”	15	0	—	”
16		”	14	0	—	”
17		”	15.5	0	—	”
18		”	15	0	—	”
19		”	12.5	0	—	”
20		”	15	0	—	”
21	Terramycin	”	14.5	0	—	”
22	15 mg twice daily,	”	16	<1	—	”
23	8 days	”	15.5	0	—	”
24		”	15.5	0	—	”
25		”	14	0	—	”
26		”	16	<1	—	”
27		”	15	<1	—	”
28		”	15	0	—	”
29		”	16	0	—	”
30		”	16	0	—	”

* S—Survived.

for this type of experiment. None of the 10 rats treated with aureomycin, and none of the 10 rats receiving terramycin showed clinical evidence of illness or significant lowering of the haemoglobin level. Bartonellae were not found in the blood of 17 of these 20 treated rats, and were present in only very small numbers in the other 3.

Discussion. Mayer, Borchardt, and Kikuth (4) found that organic arsenical compounds, including neoarsphenamine, were chemotherapeutically effective in murine bartonellosis. These agents have not given favorable results in human bartonellosis (4). Whether aureomycin or terramycin will prove to be of value in the human disease remains to be seen. It should be noted that chloramphenicol, because of its low solubility, was perhaps not given a fair trial in the experiment described above. It has been called to our attention that Dr. E. H. Payne reported the successful use of this compound in human bartonellosis to the American Society of Tropical Medicine in November, 1949.

Conclusions. Both aureomycin and terramycin, started 48 to 72 hours before the expected onset of illness and continued for 9 days were found to be effective in preventing murine bartonellosis. Rats treated with these compounds developed no evidence of illness while the morbidity in the control groups was 100%, and the mortality 50 to 100%. A few bartonellae appeared in the blood of some of the treated rats, but anemia and hemoglobinuria were prevented. The chemoprophylactic effectiveness of these 2 compounds in murine bartonellosis is in contrast to negative results obtained with a wide variety of other antibiotics.

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Chemotherapy of Leukemia. V. Effects of 2,4,6-Triethylenimino-S-Triazine and Related Compounds on Transplanted Mouse Leukemia.* (18022)

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Certain ethylenimine and triazine derivatives have been of interest to the textile industry because of their chemical reactivity(1). The structural relationship of the ethylenimine groups to transformation products of the hydrolysis of nitrogen mustards and of the triazines to the pyrimidine moiety of pteroylglutamic acid suggested an evaluation of their chemotherapeutic activity in experimental cancer. Early in the study of this group one compound, 2,4,6-triethylenimino-*s-triazine* (SK 1133), demonstrated marked activity in prolonging the survival time of leukemic mice(2). Other investigators working independently have reported a retardation of tumor growth in mice by this compound(3). It has also been shown to inhibit various solid tumors in mice and rats(4,5) and to be active against mouse tumor explanted to the chorioallantoic membrane of the chick embryo (6). Since the activity of this compound might be attributed to either its triazine or ethylenimine ring structure, compounds possessing one or the other of these were tested in an attempt to demonstrate the active moiety of SK 1133. The results of these studies are herewith presented.

Methods. The technic for the evaluation of the chemotherapeutic activity of a given drug by means of its ability to prolong the survival time of mice with transmitted leukemia has been described previously(7,8).

Leukemia Ak4(9), a relatively acute strain, was used in these particular experiments. In a typical experiment, 240 mice of the inbred Akm stock were injected intraperitoneally with 0.1 cc of a saline suspension containing 1,000,000 cells. Forty-eight hours after injection these mice were divided into comparable groups of ten mice each (two sets of untreated controls, two sets of controls treated with a standard compound of known activity, 4-amino-N^{10}-methyl-pteroylglutamic acid, and twenty sets of mice treated with unknown compounds). Compounds were given intraperitoneally in maximum tolerated doses 3 times weekly for 10 doses. Water soluble compounds were dissolved in saline. Substances insoluble in water were usually suspended in 5% gum arabic in saline. The results of treatment with an unknown substance were compared with those obtained with the standard compound, 4-amino-N^{10}-methyl-pteroylglutamic acid, which has previously been shown to possess a high degree of chemotherapeutic activity against Ak4 leukemia(8,10). Maximum tolerated dosage was generally used throughout in an attempt

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TABLE I.
Effect of SK 1133 (A) on Transmitted Mouse Leukemia Ak 4.

Dose, mg/kg	Weight change		Untreated		Treated		
	Untreated*	Treated†	No. mice	Avg survival time (days)	No. mice	Avg survival time (days)	% increase
One gum arabic suspension used throughout entire course of injections.							
4	4.2	—0.2*	20	9.6	10	14.7	53
4	2.3	—3.2	19	13.3	6	26.8	101
4	0.8	2.4	20	11.7	9	25.2	115
4	2.5	—1.6*	19	11.7	9	14.8	26
4	1.8	—0.9	19	9.3	20	15.4	66
4	1.6	—1.9	20	9.6	10	15.5	62
2x1 4x9	—0.5	—1.4	20	11.4	9	26.1	129
2x1 4x9	1.7	—0.9	18	12.8	10	19.0	48
Gum arabic suspension prepared 24 hr before first inj. with same suspension used throughout entire course of inj.							
8	.7	—2.9	15	11.3	18	19.9	76
4	.7	0.3*	15	11.3	18	16.3	44
2	1.7	1.4*	18	11.9	10	12.9	8
Gum arabic suspensions prepared before each inj.							
1.5	1.5	—0.2	19	13.5	9	22.5	67
Saline suspension prepared before each inj.							
.75	4.7	—0.4*	18	10.2	10	17.0	67
.60	1.1	—2.9*	9	9.6	9	16.8	75
.55	2.1	0.6*	18	9.8	8	13.0	33
Filtered portion of saline suspension used throughout entire course of inj.							
1.0	1.9	—1.3	19	11.6	8	22.0	90
0.5	1.9	2.0*	19	11.6	10	12.8	10

* Wt change calculated as difference between initial wt and that one week later.
† Wt change calculated as difference between the initial wt and that 2 weeks later.

to procure the maximum effect, but in some experiments lower doses were employed. The mice were observed for the development of leukemia and autopsied at death. If gross evidence of leukemia was not conclusive, microscopic sections were taken.

Results. The results of the regular screening experiments with SK 1133 are presented in Table I. The drug was initially suspended in 5% gum arabic in saline. Later the possibility of a reaction between the gum arabic and SK 1133 was considered and tests were made of the activity of the compound suspended in saline. By this technic considerably higher toxicity was noted and smaller doses showed therapeutic effect. Later studies done by the Pharmacology Section(11) demonstrated that the insoluble fraction of the drug was only 2% by weight and that a filtrate of SK 1133 in saline retained the full toxicity of the original suspension. Although higher doses of the gum arabic suspensions had to be given, definite activity was shown by gum arabic suspensions, saline suspensions, or saline solutions when the maximum tolerated dose was employed. At half this dosage level very little effect was noted from the agent in any vehicle. At no tolerated dose was it as effective as 4-amino-N^{10}-methyl-PGA (Fig. 1).

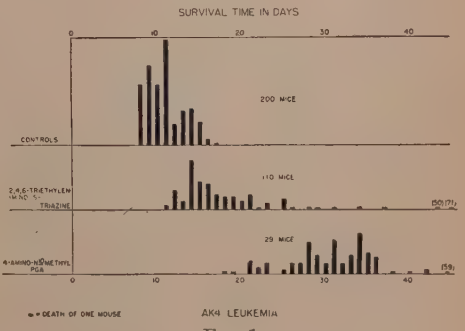

FIG. 1.

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TABLE II.
Effect of Certain Triazines and Ethylenimines on Transmitted Mouse Leukemia Ak 4.

Compound	Source of supply	Dose, mg/kg	Weight change, g		Untreated		Treated		
			Untreated*	Treated*	No. mice	Avg survival (days)	No. mice	Avg survival (days)	% increase
2,4-Diethylenimino-6-amino-*s*-triazine	A	.35§	2.1	—2.9†	18	9.8	9	18.6	90
		.35§	1.6	0.7	8	9.1	10	14.2	56
		.40§	1.6	—3.7†	20	9.6	7	25.1	161
		.40§	1.8	—2.2	9	8.9	9	16.5	85
2,4-Diethylenimino-6-phenyl-*s*-triazine	A	30	1.7	—0.3	18	11.5	8	13.3	16
		25§	2.2	1.6	17	9.0	9	10.3	14
		30x1§ 25x7§	1.9	0.5	20	10.7	10	13.6	27
		30‡	0.1	0	10	10.5	10	11.4	9
		35	—0.5	—0.6	19	9.4	10	12.2	30
2,4,6-Tris(dimethylamino)-*s*-triazine	A, B	125	3.9	0.2	17	13.6	9	18.6	36
		100	3.5	2.1	18	12.7	10	14.6	15
		100	2.2	—1.2	22	10.9	10	15.0	38
		50‖	2.0	2.1	10	10.6	10	12.4	17
		100§	2.2	1.1	17	9.0	10	9.6	7
Hexahydro-1,3,5-tris(methylacrylyl)-*s*-triazine	C	350¶	0.6	0.9	18	7.6	10	12.1	59
		500	1.6	1.5	8	9.1	8	11.4	25
		350	1.0	—2.4	18	10.7	8	14.0	31
Hexamethylene diethylenurea	A	10-15	0.7	—1.8†	15	11.3	7	21.3	97
		1§	—	0.5	10	7.2	10	16.1	124
		1.25§	0.1	—3.8†	10	10.9	7	16.0	47
Tolylene diethylenurea	A	1.5§	4.7	1.0	18	10.2	10	13.9	36
		2.0§	4.7	1.2	18	10.2	9	12.8	25
		3.0§	4.2	3.0	20	9.6	9	15.4	60

* Wt. change calculated as difference between initial wt. and 1 wk. later.
† 2 wks. later.
‡ Gum arabic suspension prepared before each injection.
§ Saline suspension prepared before each injection.
‖ One saline suspension used throughout entire course of inj.
¶ One propylene glycol sol. used throughout entire course of inj.

Table II lists the positive results obtained from experiments in which certain compounds containing a triazine or an ethylenimine component or both were tested for chemotherapeutic activity. Unless otherwise noted, one gum arabic suspension was used throughout the entire course of injections. On each compound tested the dosage was chosen on the basis of preliminary toxicity studies conducted in the Department of Pharmacology at Sloan-Kettering Institute under the direction of Dr. Frederick S. Philips. The letters listed beside each compound indicate the sources from which the materials were obtained for these experimental studies.†

The following chemically related compounds were also tested and they showed no significant effect against transmitted leukemia Ak4: Octadecylethylenurea, 2,4-Diamino-6-hydroxy-*s*-triazine, (supplied by A); 2,4-Diamino-6-n-propoxy-*s*-triazine, 2,4-Diamino-6 - (2′-β-hydroxy-ethoxy-5′-arsonoanilino) -*s*-triazine, 2,4-Diamino-6-[4-*bis*(carboxy-methylenethio)arsenisoanilino]-*s*-triazine, 2,4-Diamino - 6 - (5′-arsono-2′-hydroxyanilino) - *s*-triazine, 2-Amino-4-morpholino-6-chloro-*s*-triazine, 2-Amino-4-cyclohexylamino-6-chloro-*s*-triazine, 2-Amino-4-N-propylamino-6-chloro-*s*-triazine, 2-Amino-4-methylamino-6-chloro-*s*-triazine, 2-Amino-4-ethylamino-6-chloro-*s*-triazine, 2-Amino-4-diallylamino-6-chloro-*s*-triazine, 2-Amino-4-(β-hydroxyethyl-ethylamino) -6-chloro-*s*-triazine, 2-Amino-4-(N-β-hydroxy-ethylanilino)-6-chloro-*s*-triazine, 2,4-Bis-methallylamino-6-chloro-*s*-triazine, 2,4-Bis(dimethallylamino)-6-chloro-*s*-triazine, 2,4-Bis(β-hydroxypropylamino)-6-(4′-arsonoanilino)-*s*-triazine, 2,4-Diallylamino-6-chloro-*s*-triazine, 2 - Methallylamino - 4 - dimethallylamino - 6 - chloro - *s* - triazine, 2 - Dimethylamino - 4,6 - dichloro-*s*-triazine, 2-Ethylamino-4-diethylamino - 6 - chloro - *s* - triazine, 2,4 - Dipiperidino -6-chloro-*s*-triazine, (supplied by B); Hexahydro-1,3,5-triacrylyl-*s*-triazine, (supplied by C); 2,4-Bis(hydroxymethylamino)-6-(α-hydroxymethyl)-benzyl-*s*-triazine, (supplied by D); 2,4-Diamino-6-benzyl-*s*-triazine, 2-Amino-4,6-diacetamido-*s*-triazine, (supplied by E); 2,4-Diamino-*s*-triazine, 2,4-Diamino-6 (β-dimethylamino-ethoxy)-*s*-triazine, (supplied by F); 2,4-Bis(aminomethylamino)-6-bis(aminomethyl)amino-*s*-triazine, (supplied by E and H); 2,4,6-Triamino-*s*-triazine, (supplied by A and F); 2,4,6-Trihydroxy-*s*-triazine, (supplied by B, C, and G); 2,4,6-Trimethylolamino-*s*-triazine, (supplied by A and I); 2,4-Diamino-6-chloro-*s*-triazine, (supplied by F and G); 2,4-Diamino-6-mercapto-*s*-triazine, (supplied by D, E, and B); 2,4-Diamino-6-(4′arsonoanilino)-*s*-triazine, (supplied by B and G).

Discussion. The results of these experiments demonstrate that in this series the compounds showing the ability to prolong significantly the survival time of mice with transplanted leukemia Ak4 all possess at least 2 ethylenimine rings. The possession of a triazine or melamine moiety by the active agent does not seem to be essential since one compound, hexamethylene diethylenurea, tested in this series could not be classified as containing either of these groups but appeared to possess definite activity by virtue of the ethylenimine rings. In these studies none of the compounds possessing a single ethylenimine ring prolonged significantly the survival time of leukemic mice. This is analogous to the results obtained against transmitted mouse leukemia with the nitrogen mustards (7) where no agent had chemotherapeutic effect against leukemia unless it possessed at least 2 beta halogenated alkyl groups. In studies on the effect of this type of compound on the leukocyte count of the leukemic mouse(12), however, ethylenimine and 2,4-dimethoxy-6-ethylenimino-*s*-triazine, as well

† We wish to acknowledge at this time the generosity of the following groups in supplying the compounds used in these studies:
A—Calco Chemical Co.
B—Parke, Davis and Co.
C—Goodrich Chemical Co.
D—Abbott Laboratories.
E—Monsanto Chemical Co.
F—G. D. Searle and Co.
G—Dr. Ernst. A. H. Friedheim.
H—Chemical-Biological Coordination Center of the National Research Council.
I—Imperial Chemical Industries, Ltd.

12. Burchenal, J. H., Biedler, J. L., Meigs, G. M., and Nutting, J., unpublished data.

as the compounds mentioned here as showing therapeutic activity, caused a definite fall in the leukemic leukocytes in the peripheral blood. Clinical trials with SK 1133 (triethylene melamine) have shown it to possess chemotherapeutic activity similar to that of methylbis(β-chloroethyl)amine against Hodgkin's disease, lymphosarcoma, and the chronic leukemias(13). It would seem valid, therefore, to attribute the activity as chemotherapeutic agents, of the particular series of compounds discussed in this report, to the nitrogen mustard-like ethylenimine ring rather than to the triazine or melamine structure.

13. Karnofsky, D. A., Burchenal, J. H., Southam, C. M., Bernstein, J. L., Armistead, G. C., Craver, L. C., and Rhoads, C. P., unpublished data.

Summary. 1. 42 compounds, of which 39 could be classed as triazines and 3 as ethylenimine ring compounds were screened for chemotherapeutic activity against Ak4 mouse leukemia. 2. 2,4,6-triethylenimino-*s*-triazine, 2, 4 - diethylenimino - 6 - amino - *s* - triazine, and hexamethylene diethylenurea significantly prolonged the survival time of mice injected with transplanted leukemia Ak4. 3. It would appear that the chemotherapeutic activity of the above compounds can be attributed to the ethylenimine structure. 4. Four other compounds containing either a triazine or an ethylenimine moiety showed a slight chemotherapeutic effect.

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Inactivation of Estradiol by the Hepatic Tissues of Mice.* (18023)

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Because of the role that estrogens play in experimental carcinogenesis and in many clinical conditions, considerable significance is attached to any factor which would increase or decrease the titer of circulating estrogens. The liver destroys estrogen in a majority of laboratory animals(1-4). Marked damage to the liver in these animals caused a rise in titer of the circulating estrogen(5,6). Factors which will decrease the liver's ability to inactivate estrogen include carbon tetrachloride toxicity(5), virus hepatitis(7), partial hepatectomy(6), and nutritional deficiency(8,9). An important aspect of this problem is: what is the effect of large quantities of estrogen in the blood upon the liver's ability to inactivate estrogen?

The effect of a prolonged castrate condition and of prolonged hyperestrogenemia upon the ability of the mouse's liver to inactivate estrogen was studied.

Methods. Animals used were first generation hybrid mice, designated CC_1, CC_2, AC_1, AC_2, AC_3, and AC_4. A few inbred mice of the C_{57}, NHO and CHI strains were used. The mice were divided into 3 groups, one group was untreated, mice of the second group were castrated, and mice of the third group were castrated and then 2 mg pellets of estrone were implanted subcutaneously. Six weeks, 8

* This investigation was aided by grants from the James Hudson Brown Memorial Fund of the Yale University School of Medicine, and the United States Public Health Service (administered by W. U. Gardner).

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TABLE I.
Hepatic Inactivation of Estradiol in Mice.

Condition of animals	Substrate	No. of animals	Mean age (mo.)	Mean QO_2	Amt of estradiol inactivated, m.u./mg dry wt
Normals	Without estradiol	7	2½	5.1 ± 1.20	—
"	With "	13	2½	5.1 ± 0.83	8.5 ± 1.05
Castrated 6 wks	" "	5	3½	5.2 ± 0.73	5.6 ± 0.88
Castrated and Imp.* 6 wks	" "	5	3½	5.4 ± 0.71	5.5 ± 0.95
Castrated 8 mo.	" "	8	12	4.7 ± 0.83	6.3 ± 1.05
Cast. and Imp. 8 mo.	" "	8	11½	5.4 ± 0.59	6.6 ± 0.84
Castrated 18 mo.	" "	4	21½	5.3 ± 0.54	6.5 ± 1.14

* Imp.—refers to implantation of pellets of estrone.

months, and 18 months following castration or castration and the implantation of pellets the mice were killed, their livers removed, and the activity of their hepatic tissues in inactivating estradiol was tested. Thin slices of liver were cut and placed on a solution containing a known amount of estradiol in a Warburg flask for a period of one hour(10). The flasks were then placed in boiling water for 10 minutes to destroy enzymatic activity. The amount of estradiol remaining at the end of one hour was then estimated by bio-assay (11). The estradiol was dissolved in a Krebs-Ringer-phosphate buffer solution(12). The liver was sliced between 0.4 and 0.6 mm in thickness, a size which is optimal for hepatic tissue used in manometric studies(13). The Warburg flasks were shaken at a rate of one hundred times per minute and maintained at a temperature of 37.5° ± 0.02°C.

All assays were carried out by the vaginal smear method. A total of 12 or more mice were used for each assay. The entire contents of the Warburg flasks, including the liver slices, were homogenized and injected in fine suspension.

Results. The results of these experiments may be appraised by referring to the accompanying table and graph. In order to determine whether hepatic tissue has any estrogenic activity, seven experiments were performed in which homogenized hepatic tissue from mice was bio-assayed. Amounts of mouse's liver ranging from 12.0 mg to 39.0 mg showed no estrogenic activity in these bio-assays.

Mice which were castrated and implanted with pellets of estrone lost an average of 3 g in weight. Mice which were castrated but not implanted with estrogen gained an average of 4 g before they were sacrificed.

Discussion and conclusion. The incubation of mouse's liver with estradiol did not raise the Qo_2 of the hepatic tissue. Moreover a decrease in the ability of the hepatic tissue from mice to inactivate estradiol *in vitro* was not accompanied by a change in the Qo_2 of the liver slices. Although the inactivation of estradiol is generally thought to be an oxidation reaction(14) the oxygen uptake of the inactivation reaction was too small to be detected by the Warburg technic.

One milligram by dry weight of mouse's liver inactivated 8.5 mouse units of estradiol in one hour under the experimental conditions used. This figure represents the maximum amount of estradiol that one milligram of mouse's liver inactivated. If less than this concentration of estradiol was present, all the estradiol present was inactivated. If more than 8.5 m. u. of estradiol per milligram of hepatic tissue was present, increasing amounts of estradiol remained unchanged in solution as the concentration of estradiol was increased.

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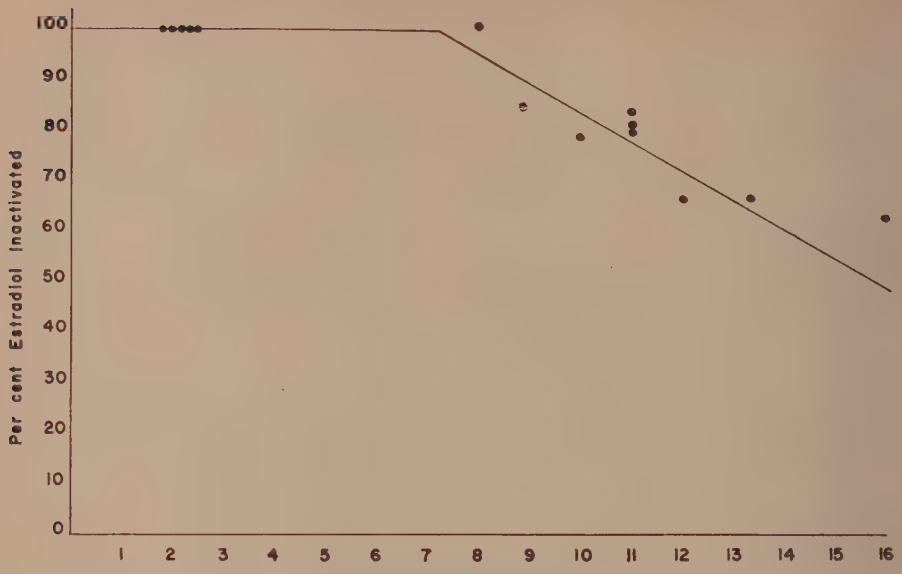

FIG. 1.

The maximum rate of inactivation of estradiol by hepatic tissue in these experiments was thus unaffected by the concentration of estradiol present (see graph).

The capacity of liver slices from castrated mice to inactivate estradiol was significantly below that of intact, untreated mice. This fall in inactivating capacity was observed within 6 weeks after operation and was maintained for 18 months. Slices of liver from mice that were castrated and received pellets of estrone showed a decrease in estradiol inactivating capacity similar to that found in castrated mice. It would seem from this that the fall in inactivating capacity of the mouse's hepatic tissue was the result of castration alone and that the addition of pellets of estrone did not alter the change. On the other hand, it is possible that the animals implanted with pellets of estrone achieved the changes in hepatic inactivation through a somewhat different mechanism.

Weight loss had little effect on the loss of estradiol inactivating ability of the hepatic tissue. The same changes in hepatic inactivation of estradiol were observed in the livers of mice which were castrated and gained weight and in the livers of the mice which were castrated and implanted with estrone and lost weight.

Summary. Hepatic tissue of normal mice inactivated 8.5 m.u. of estradiol per mg dry weight per hour. Hepatic tissue from castrated mice or from estrone-treated mice was slightly less effective.

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Ethylenedinitrilotetraacetic Acid as a Solvent for Urinary Calculi. (18024)

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Ethylenedinitrilotetraacetic acid is now available commercially from Bersworth Chemical Co., Framingham, Mass. It forms stable chelate complexes with a number of metal ions, including calcium(1). We find that neutral aqueous solutions of this reagent dissolve urinary calculi rapidly. Urinary calculi are composed principally of calcium oxalate, urate, phosphate, and carbonate, with other salts and organic matter(2). The solvent action of ethylenedinitrilotetraacetic acid is undoubtedly due to the formation of soluble chelated calcium complexes. One stone which was composed of cystine, with no calcium present, was unaffected by the acid. Because of its effect on urinary calculi, we propose the short name "calsol" for ethylenedinitrilotetraacetic acid in this application.

A 3% calsol solution was made up by dissolving 30 g of calsol and 10 g of sodium hydroxide in one liter of water and adjusting to pH 7.5 with additional sodium hydroxide. Approximately 2 g was required. For the purpose of comparing calsol with the currently used citrate solution of Suby, 3% sodium citrate was prepared according to his formula "G"(3). The solvent actions of these solutions were compared against 44 pairs of stones from 44 patients in the Squier Urological Clinic, Presbyterian Hospital, New York. The stones from any one patient were nearly identical in size and composition. They were classified into 3 types according to the principal anion present: oxalate, urate, and soft (phosphate-carbonate). Calcium was present in all stones, including the urates. The stones were individually immersed in the solutions for 24 hours and the percentage losses in weight determined. Care was taken to avoid breaking off small pieces of stones which had been softened by the solution. In each pair of stones the calsol-treated stone lost substantially more weight than the citrate-treated stone. Results are summarized in Table I.

The effects of wide variations in physical and chemical structure of the stones is evident, even when the stones are classified according to chemical type. The citrate solutions show satisfactory solvent action against only the most soluble phosphate-carbonate type, in accordance with the results reported by Suby *et al.*(3). The calsol solutions, however, dissolve substantially all types of stones. We are completing an intensive laboratory and clinical study of calsol solutions.

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TABLE I.
Dissolution of Urinary Calculi in 24 Hr by Calsol and by Citrate Solutions.

		Wt loss in %			
		3% Calsol (pH 7.5)		3% Citrate (pH 4)	
Type of stone	No. of specimens	Avg	Range	Avg	Range
Oxalate	8	27	11-45	2	0-6
Urate	15	16	5-29	3	0-17
Soft	21	34	7-86	17	0-50

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Glomerular Filtration Rate and Renal Plasma Flow During Mannitol Loading in Hydropenic Dogs. (18025)

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Data are presented on the glomerular filtration rate and renal plasma flow of hydropenic dogs before and during osmotic diuresis induced by mannitol loading.

Experimental. The experiments were performed on trained, unanesthetized animals maintained on a diet of commercial dog food and horse meat, deprived of water for 20 hours and of food for 16 hours prior to experimentation. A detailed description of the procedure will be published elsewhere(1). Clearances of creatinine and para-aminohippurate were determined for 2 periods prior to and for 6 periods after mannitol loading. The priming and maintenance doses of creatinine and sodium para-aminohippurate were administered intravenously in hypertonic solution. During the first 3 post loading periods plasma mannitol was maintained at about 30, and, during the last 3, at about 60 milliosmols per liter. The analytical methods have been described previously(2).

Results. The results of these experiments are given in Table I. For the sake of brevity only one pre-loading and 2 post-loading periods are listed. The data include concurrent time, urine flow, and the clearance of creatinine, paraaminohippurate, mannitol and urea.

Although urine flow increased as much as 28 fold after loading, no consistent change in creatinine clearance, renal plasma flow or filtration fraction occurred. The creatinine clearance increased in one experiment and diminished slightly in two, while the renal plasma flow increased slightly in all 3. None of the measurements changed by more than 15% of their preinjection value. The filtration fraction fell in 2 experiments and increased in a third.

The mannitol clearance ranged from 0.89 to 1.12 of the creatinine values.

The urea clearance increased markedly after loading. The ratio of urea to creatinine clearance during preliminary periods varied from 0.35 to 0.40, increasing to as much as 0.90 after loading. Similar ratios were obtained by Shannon(3) during osmotic diuresis.

TABLE I.
Clearance of Urea, Mannitol, Creatinine, and Para-aminohippurate Before and During Osmotic Diuresis Produced by Mannitol Loading.
The values are expressed in cc/min/M^2.

Period	Concurrent time, min.	Urine flow	C_{urea}	$C_{man.}$	$C_{creat.}$	C_{PAH}	F.F. $\frac{C_{creat.}}{C_{PAH}}$
		Dog Pe. 15.5 kg, 0.62M^2.					
P-2	—30 to —4	0.29	26.1		64.7	205	.32
	0 to 9	79 cc 25% Mannitol. Maintenance 0.95 cc/min.					
1	20 to 34	4.47	46.0	65.9	73.5	215	.34
2	34 to 49	4.78	47.5	73.6	78.5	251	.31
		Dog De. 16.6 kg, 0.64M^2.					
P-2	—32 to —5	0.30	28.9		82.4	267	.31
	0 to 11	106 cc 25% Mannitol. Maintenance 0.91 cc/min.					
1	14 to 28	8.63	49.9	72.6	79.3	273	.29
2	28 to 47	6.32	47.2	61.8	69.1	241	.29
		Dog Vi. 14.0 kg, 0.56M^2.					
P-2	—44 to —11	0.34	24.8		63.9	199	.32
	0 to 8	72 cc 25% Mannitol. Maintenance 0.92 cc/min.					
1	13 to 33	5.59	55.7	69.7	62.0	227	.27
2	33 to 52	4.95	45.0	60.5	54.6	207	.26

Discussion. Data in the literature are consonant with the present studies. The creatinine clearance of non-hydropenic dogs has been shown to be unaffected by the infusion of hypertonic glucose(3) or urea(4) solutions. Other studies(5) showed similar results with sucrose and mannitol loading. The renal plasma flow was not determined in the experiments cited.

Summary. Osmotic diuresis produced by mannitol loading in hydropenic dogs does not significantly affect the glomerular filtration rate, renal plasma flow or filtration fraction.

* Senior Fellow, National Research Council.

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Electron Microscope Studies on Structure of Mitotic Figure.* (18026)

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The structure of the amphiaster has long been a subject of debate [Wilson(1) and Schrader(2).] The majority of evidence covered in these reviews indicates that the spindle is composed of fibers. The electron micrographs presented here also show the fibrillar structures in the spindle. The terminology here used in reference to the spindle components is that recommended by Schrader (2).

Material and methods. The material is from a crayfish testis, actively forming germ cells. It was fixed in Bouin's solution, embedded in a mixture of 60% paraffin (melting point, 70°C) and 40% beeswax and sectioned at 0.5μ with a Spencer No. 820 microtome equipped with a Spencer thin section adapter. The electron microscope used was an R.C.A. model E.M.U. equipped with an unbiased gun. The magnification of the figures may be determined from the micron scale appearing on each figure.

We wish to emphasize that since the cells here studied were fixed and dried the element of artifact must be kept in mind in analyzing the pictures.

Results and discussion. A portion of one-half of a longitudinal section of a metaphase spindle is shown in Fig. 1. The shape of the mitotic figure is typical of that seen in the usual preparations studied by the light microscope. The "chromosomal fibers" are about 1500-2500 Å in width. The fiber on the right side of the figure appears to be split, suggesting that the large spindle fibers in the crayfish are compound. Further evidence that the fibers are compound, *i.e.*, composed of many smaller fibers, is illustrated in Fig. 4, 5 and 8. The tangential section of the large "chromosomal fibers" in Fig. 4 clearly shows the cut ends of the smaller fibers within the compound larger ones. In addition, an apparent lateral stretching of the large "chromosomal fibers" has separated their small component fibers, in Fig. 5 and 8. The smaller fibers are about 300-600 Å in width. Some of the smaller fibers show irregular banding or beading which possibly is the result of irregular contraction at the time of fixation. Similar phenomena may also be

* We are indebted to the General Biological Supply House, Inc., Chicago, for kindly supplying us with the fixed crayfish testis.

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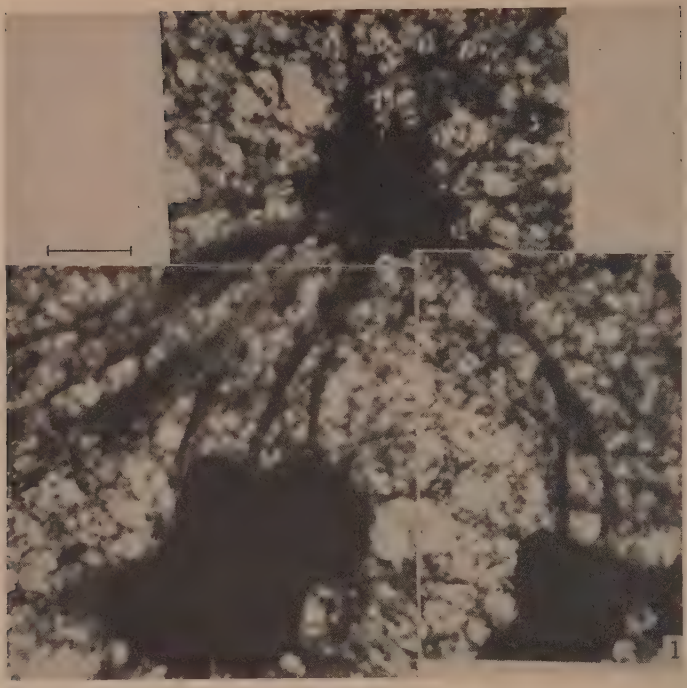

FIG. 1.
Portion of half of a longitudinal section of a metaphase spindle showing centrosome, chromosomes, "chromosomal fibers," and astral rays.

seen in the large "chromosomal fibers" of Fig. 2 and 3. In both cases the banding seems too irregular and too gross to represent periodic banding of their ultrastructure. We have not been able to identify clearly the "continuous fibers" (those extending from centrosome to centrosome) in this material. If present, they seem to be relatively small and few in number.

The centrioles are relatively dense and somewhat irregular in outline (Fig. 1, and 7). In Fig. 1 the section seems to have been through the middle of the centriole. Here short tongues of centriole material extend out along the base of the spindle fibers. In Fig. 7 the section through the centriole seems to have occurred near the periphery. This figure shows how the "chromosomal fibers" lose their orientation at the periphery of the centriole, and it indicates that the centriole is probably not a homogeneous body since varied darker and lighter areas appear within them.

Small astral rays are present about the periphery of the centriole (Fig. 1 and 7). They seem to be fibrous rather than hollow tubes. The asters in the crayfish are not as suitable for study of their structure as are those in the dividing whitefish blastula (unpublished).

A section through the interzonal region of an anaphase figure is shown in Fig. 6. Some orientation of small fibers appears between the chromosomes. These we are interpreting as the "interzonal fibers." Since the fibers here appear to be discontinuous in spots it is likely that a disorganization of them in this region is occurring. The net-like strands of protoplasm are seen extending between the larger longitudinally oriented "interzonal fibers."

The cytoplasm has the appearance of a fibrous sponge-like reticulum. This condition probably represents mainly the coagulated protein framework.

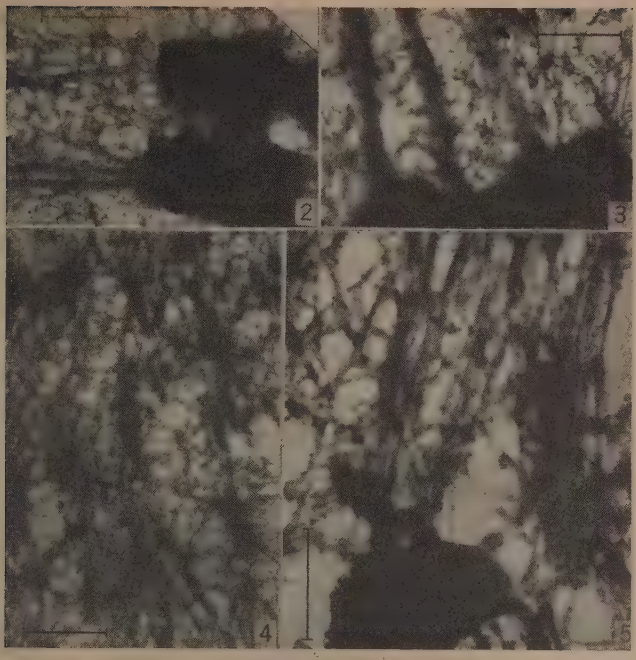

FIG. 2. Slightly laterally stretched "chromosomal fibers" with some banding in the lower fiber.

FIG. 3. Banding apparent in some "chromosomal fibers."

FIG. 4. Tangential section of "chromosomal fibers" showing cut ends of their smaller component fibers.

FIG. 5. Laterally distorted "chromosomal fibers."

As previously mentioned the method of preparation of this material has undoubtedly induced some degree of artifact. However, from the abundance of previous work on this subject with the light microscope (Schrader (2), it seems safe to conclude that for the most part the present figures of the centrosomes, "chromosomal fibers," "interzonal fibers" and astral rays represent at least roughly the condition in the living cell.

We were not able to differentiate between first spermatocyte and second spermatocyte divisions in our figures. However, from the work of Fasten(3) the appearance of the spindle under the light microscope is similar in both divisions.

Summary. The metaphase spindle of the maturation division in the crayfish testis seems to be composed largely of longitudinally oriented "chromosomal fibers" measuring about 1500-2500 Å in width. These fibers appear to be compound, *i.e.*, made up of smaller fibers of about 300-600 Å in width. Both the "chromosomal fibers" and their smaller fibrous components may at times show banding, which is possibly the result of irregular contraction during fixation. The centrioles appear relatively dense, are irregular in outline, and are probably not homogeneous bodies. The "chromosomal fibers" and astral rays lose their usual orientation at the periphery of the centriole. "Interzonal fibers" are relatively small in the late anaphase stages of the crayfish testis. Though "continuous fibers" were not clearly observed, we do not wish to claim that such fibers may not exist. Astral rays are composed of fibers, oriented as described in the past. The coagulated cytoplasm presents the appearance of a sponge-like reticulum, which is probably mostly a protein "framework," since

3. Fasten, N. I., *J. Morph.*, 1914, v25, 587.

FIG. 6. Interzonal region of anaphase, with some orientation of "interzonal fibers." The lower print which is slightly out of focus does not exactly unite with the one above.

FIG. 7. Section through portion of a centrosome.

FIG. 8. Laterally stretched "chromosomal fibers" illustrating their compound nature.

much of the other materials had been removed by the method of preparation.

We wish to express our appreciation to C. D. Janney for technical advice and assistance.

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Influence of Rice and Purified Diets upon Cardiac Behavior of Thiamine Deficient Rats. (18027)

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(Introduced by W. H. Sebrell, Jr.)

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Marked cardiac damage has been reported in severe chronic thiamine deficiency in dogs by Porto and de Soldati(1), in rats by Lowry *et al.*(2), and by Ashburn and Lowry(3), in pigs by Follis *et al.*(4), and in humans by Weiss and Wilkins(5). Aalsmeer and Wenckebach(6) have reported mild, perhaps nonspecific, heart damage in human beriberi in the Orient as shown by electrocardiographic and microscopic studies. In this country, however, Weiss and Wilkins(5) found severe cardiac damage in human beriberi patients as reflected in electrocardiographic and histologic studies. The beriberi reported by Weiss and Wilkins(5) in this country occurred mainly in alcoholics while that in the Orient occurred chiefly in persons subsisting largely on rice. This difference in the severity of cardiac involvement suggested the possibility that rice might possess some substance or property that might protect the heart. This possibility was given added impetus by the work of Kempner(7), who reported beneficial effects in hypertensive patients on a high rice diet supplemented with adequate vitamins. In order to test the hypothesis that rice might possess a protective substance or property, rats were made severely and chronically thiamine deficient by diets composed principally of rice and by other diets made of purified ingredients. The influence of each diet was ascertained by the extent of cardiac damage as shown by the electrocardiograms and pathologic examination.

Methods and procedure. Weanling male albino rats of Osborne and Mendel or Sprague-Dawley (National Institutes of Health Colony) strains were housed individually in elevated wire bottom cages. The animals were divided into groups and were fed the following diets *ad libitum*:

Group	No. of rats	Diet
I	10	2293 (purified diet with adequate thiamine)
	20	2292 (purified diet with no thiamine)
II	5	2369 (rice diet with adequate thiamine)
	20	2299 (rice diet)
III	20	2619 (purified diet with thiamine added approximately equal to rice diet 2299)

Rats receiving diets 2293, 2299, and 2369 were comparable as to age, weight and litter and were started on the diets when approximately 50 g in weight. Rats receiving diets 2292 and 2619 were maintained on a stock diet until approximately 110 g in weight after which the experimental diets were started. Preliminary experiments showed that this was necessary to produce rats which were comparable in body size and general condition at the onset of thiamine deficiency. The composition of the diets is given in Table I. The two lots of rice used in this experiment contained 61 μg and 64 μg of thiamine per

* Part of this work was submitted to the Faculty of the Graduate Council of the George Washington University in partial satisfaction of the requirement for the degree of Doctor of Philosophy.

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2. Lowry, J. V., Sebrell, W. H., Daft, F. S., and Ashburn, L. L., *J. Nutrition*, 1942, v24, 73.

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4. Follis, R. H., Jr., Miller, M. H., Wintrobe, M. M., and Stein, H. J., *Am. J. Path.*, 1943, v19, 341.

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6. Aalsmeer, W. C., and Wenckebach, K. F., Wien, *Arch. f. inn. Med.*, 1929, v16, 193.

7. Kempner, W., *North Carolina M. J.*, 1944, v5, 125.

TABLE I.
Composition of Test Diets.

Diet 2292		Diet 2299		Vitamin Powder	
				mg per g	
Casein (Vit. free)	18	Casein (Vit. free)	12.1	Thiamine	0
Sucrose	72	Rice (40-60 mesh)	77.9	Riboflavin	.3
Cottonseed Oil	3	Cottonseed Oil	3.0	Pyridoxine HCl	.25
Cod Liver Oil	2	Cod Liver Oil	2.0	Nicotinic Acid	2.0
Osborne and Mendel salt mixture(9)	4	Osborne and Mendel salt mixture(9)	4.0	Ca Pantothenate	2.0
Vit. Powder	1	Vitamin Powder	1.0	Choline Cl	100.0
				Inositol	10.0
				Vit. K (menadione)	.1
Diet 2293 = Diet 2292 + 200 μg thiamine/100 g				Biotin	.01
" 2619 = " 2292 + 39 " " "				Folic Acid	.1
" 2369 = " 2299 + 200 " " "					

Alpha-tocopherol (3 mg) was given by supplement dish once a week. Vit. A and D were supplied in the diet by the cod liver oil, each gram of which supplied at least 850 and 85 USPXI units of Vit. A and D, respectively.

100 g as determined by fluorometric assay(8). A rice diet completely free of thiamine would have been desirable in these experiments, but pilot studies revealed that rice autoclaved at 250°F (15 lb pressure) for 6 hours to destroy the thiamine, underwent changes which seriously altered its nutritional quality. However, a diet composed of 77.9% natural white polished rice and 12.1% casein supplemented with fat and with vitamins except thiamine (diet 2299) produced a severe thiamine deficiency in spite of the trace of thiamine in the rice. This deficiency could not be differentiated by ordinary signs and symptoms from that resulting from a diet almost completely free of thiamine. To control the effect of the trace of thiamine in the polished rice, a similar amount of thiamine was added to one of the purified diets. Casein was added to the rice diets to equalize the protein content.

The animals in Group I on diet 2292 received 7 μg of thiamine hydrochloride in 1 ml of water, 3 times weekly by supplement dish until the sixty-third day of the experiment at which time the thiamine was withdrawn and the first acute thiamine deficiency was allowed to follow. This was done, as in previous experiments(3), to produce a chronic thiamine deficiency and to produce a deficiency of a severity equal to that developed by rats on diets 2299 and 2619 which contained some thiamine. Acute polyneuropathy (spasticity, ataxia and convulsions) was used as the principal indication of severe thiamine deficiency. Other confirmatory signs were large weight loss, decreased heart rate, muscular weakness, apathy, lack of muscular tonus, reduced blood pressure, and cyanosis. As the animals developed acute polyneuropathy, they were treated with 50 μg of thiamine hydrochloride in 0.25 ml of water by stomach tube, and then allowed to develop a second acute deficiency, and again treated with thiamine. In a few cases this was repeated for a third and fourth deficiency. In order to emphasize any cardiac changes that might be developing, the rats were made as thiamine deficient as possible before treatment. Electrocardiograms were taken on all animals once a week, and in some cases more often. When animals showed acute thiamine deficiency symptoms, electrocardiograms were taken at frequent intervals before and after thiamine therapy.

Electrocardiograms. The electrocardiograms were obtained with a Sanborn electric "Portocardiograf" in conjunction with a Sanborn "Cardioscope" amplifier, similar to the apparatus described by Rappaport and Rappaport(10). Considerable difficulty was experienced during this experiment in securing satisfactory electrocardiograph electrodes for

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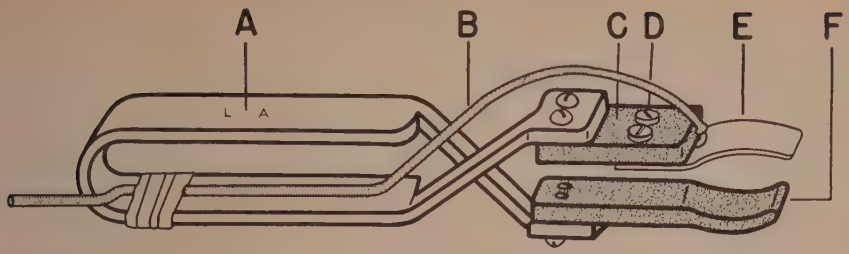

FIG. 1.
German silver electrocardiograph contact electrode.

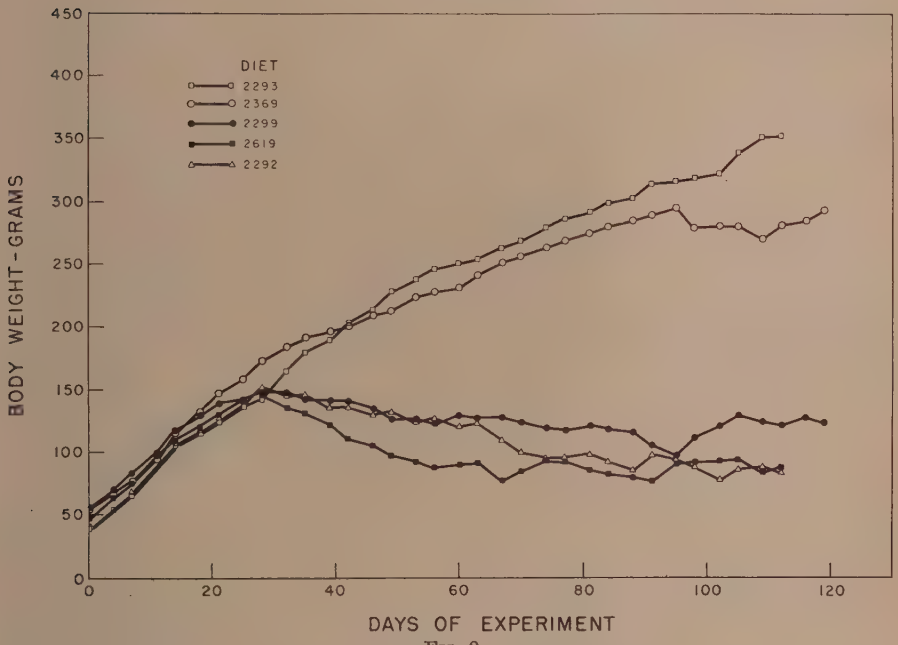

FIG. 2.
Average growth curves of rats on the test diets.

the rat. A simple German silver electrode (Fig. 1) was designed for use with the unanesthetized rat. The electrode consists of a flat spring clip (A) made of phosphor bronze (grade A, 5%, spring temper) 1/50 x ¼ x 1½ inches. To one end of the flat spring clip is attached a piece of bakelite (C) 1/16 x 1/4 x 1/2 inches to which is fastened the German silver electrode (E) 13/1000 x ¼ x ½ inches. The German silver electrode is attached to the bakelite with two German silver screws (D). The German silver used for the electrode is composed of nickel 18, copper 56, and zinc 26%. The end of the patient cable (B) is inserted between the bakelite and the German silver electrode where it is securely held in place by tightening the two German silver screws. This arrangement facilitates replacement of the cable wire whenever necessary. Soldering, which would introduce other foreign metals and possible polarization, is eliminated. The cable is made of shielded grounded copper wire (0.080 inch O.D.) of extreme flexibility and limpness, such as is used for phonograph pick-up cable. To the other end of the spring clip is attached a piece of bakelite (F) 1/16 x 1/4 x 13/16 inches. The three standard limb leads

TABLE II.
Average Heart Rates (B.P.M.) of Experimental Rats During Acute Thiamine Deficiency Episodes and Following Thiamine Therapy.

Deficiency period	Diet 2292		Diet 2299		Diet 2619	
	During	After	During	After	During	After
1	267 (19)	463 (14)	342 (18)	484 (15)	311 (14)	470 (13)
2	282 (10)	435 (8)	330 (15)	472 (11)	312 (12)	477 (9)
3	288 (7)		310 (8)		258 (6)	

Number of animals measured is indicated by figures in parentheses.

TABLE III.
Incidence of Rhythm Changes in Experimental Rats in the 3 Groups.

Deficiency episodes	Diet 2292		Diet 2299		Diet 2619	
	No. of rats	Rats with rhythm changes	No. of rats	Rats with rhythm changes	No. of rats	Rats with rhythm changes
1	19	6	19	5	20	2
2	13	11	15	5	14	2
3	7	5	10	3	11	1*

* During this deficiency period 4 rats died before tracings were obtained.

were used. The unanesthetized rat was held in its normal crouched position by the operator. A darkened quiet room was found helpful in keeping extraneous interfering stimuli at a minimum.

Results. The thiamine deficiency produced by the test diets in this study was very severe in spite of the fact that some of the diets contained a trace of thiamine. In Fig. 2 the average body weight curves for the control and experimental animals in each group are depicted. The thiamine deficient rats on the rice diet 2299 maintained their body weight at a higher level than did the thiamine deficient animals on the purified diets 2292 and 2619. The control animals on diets 2293 and 2369 grew approximately the same. Rats going through an acute thiamine deficiency episode displayed characteristic bradycardia. The animals on the rice diet (2299) had a less severe bradycardia than did the animals on the purified diet. However, a purified diet with thiamine added approximately equal to that in the rice diet also produced a less severe bradycardia. (Table II). The heart rates after thiamine therapy were similar in all the groups.

Control animals showed no electrocardiographic changes other than the slight slowing of rate associated with aging. The electrocardiograms of many of the thiamine deficient rats showed marked changes in rhythm, such as sinus arrhythmia, complete arrhythmia, A. V. block with wandering pacemaker, auricular fibrillation and sinus arrest similar in all respects to those fully described by Hundley *et al.*(11). The incidence of rhythm abnormalities was considerably less in the rats on the rice diet (2299) than on the purified diet (2292). However, rats on a purified diet with thiamine approximately equal to that in the rice diet also showed a low incidence of rhythm abnormalities (Table III).

Pathology. The pathologic lesions of the heart in these experiments were similar in all respects to those observed and fully described by Ashburn and Lowry(3). In summary, the microscopic findings were degenerative changes of muscle fibers of varying extent and severity, cellular infiltration, fibroblast proliferation or varying degrees of fibrosis. The pathologic changes were usually confined to the auricles and/or the pulmonary veins and rarely involved the ventricles.

The hearts of the control animals on the rice diet 2369 showed no pathologic changes. The hearts of two control rats on diet 2293 for some unexplainable reason showed patho-

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TABLE IV.
Incidence of Heart Lesions in Rats on the Experimental Diets.

Diet	No. of rats examined	No. of rats with heart lesions
2292	17	10
2293	19	5
2619	18	6

logic changes similar to those seen in the thiamine deficient animals. Such changes have not been observed previously in the many control rats examined in this laboratory (2,3,11). It is possible that the tissues of the control group (2293) and the experimental group (2292) were mixed during their preparation for pathologic examination. If a mix-up did occur, it could only have been between the 2292 and 2293 groups, since they were the only groups examined simultaneously. A comparison of the various groups showed that the incidence and severity of pathologic damage were less in the hearts of rats on the rice diet (2299) than in those of rats on the purified diet (2292). However, rats on the purified diet with thiamine added to equal approximately that naturally present in the rice diet, received protection similar to that exhibited by the latter diet (Table IV).

Discussion. The results of this experiment indicate that the hearts of thiamine deficient rats received more protection from a rice diet than from a purified diet. However, this protection was undoubtedly due to the trace of thiamine naturally found in rice since a purified diet containing equivalent thiamine provided about the same protection as the rice diet. It is evident that the thiamine present in the rice diet and the approximately equal amount of thiamine present in one of the purified diets was insufficient to protect against the development of rat beriberi, but was sufficient to produce a definite reduction in the incidence and severity of heart damage. This suggests that the heart can be protected against damage in beriberi by an amount of thiamine insufficient to modify the clinical disease appreciably. The fact that Aalsmeer and Wenckebach(6) observed only mild cardiac damage in their rice-eating Oriental beriberi patients while Weiss and Wilkins(5) observed severe cardiac damage in their chronic alcoholic Occidental beriberi patients, may also be explained by the small amount of thiamine available to the former group as compared to the probably almost complete lack of thiamine in the alcoholics.

The data provide no support for the possibility that rice contains a property or substance which conveys a rather general protection to the heart. However, the conditions used in these experiments produced a severe and chronic deficiency state. If tests were conducted using milder conditions or a different test system, the results might have been quite different.

The tests described in the present communication were conducted using diets in which protein, salt, vitamins and fat intake were adequate and equalized. Thus low sodium, low protein and other vitamin deficiencies should not have complicated the results. Thiamine deficient rats restrict their food intake voluntarily and thus reduce their overall intake of protein and other dietary essentials. However, previous work in this laboratory(3) using paired feeding technics has shown that inanition itself will not produce cardiac damage if the thiamine intake is adequate. What effect rice might have had if the diets were also low in protein, fat and salt, as in the Kempner diet(7), is conjectural.

Summary. Cardiac damage was produced in rats by a severe chronic thiamine deficiency. Rats fed a natural polished rice diet and/or a purified diet with thiamine added to approximate that naturally present in rice had less severe physiologic and pathologic changes than rats fed a thiamine free purified diet. The results of this experiment indicate that the protection shown by the rice diet and a purified diet with thiamine approximately equal to that of the rice diet was due to the trace of thiamine and not to some unknown substance or property in rice.

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Production of Quellung Antisera in Chickens. (18028)

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The usefulness of the Neufeld capsular reaction for serological identification of certain microbial species is now generally recognized. The list of microorganisms for which preparation of type-specific antisera suitable for Quellung has been reported includes among others: *Diplococcus pneumoniae*(1), *Streptococcus salivarius*(2), *Streptococcus MG*(2), *Streptococcus agalactiae*(3), *Neisseria meningitidis* (4), *Hemophilus influenzae*(5), *Klebsiella pneumoniae*(3,6), *Bacterium antitratum*(7,8), *Sporotrichum schenckii*(9), *Cryptococcus neoformans*(10,11). In the case of the Gram-negative species, the preparation of such antisera in rabbits is often troublesome since prolonged courses of immunization may be necessary to ensure adequate antibody production and the toxicity of certain components of the bacterial cells often leads to premature death of the animals. In view of the ease with which antiserum for capsular swelling of meningococci can be obtained in chickens (12), it seemed worthwhile to attempt production of similar antisera versus *H. influenzae* type B and *K. pneumoniae* types A and B which are not uncommonly encountered in serious pathologic conditions and for which the diagnostic rabbit antisera are not always readily obtained from commercial sources.

Materials and methods. Preparation of Antigens for Injection. K. pneumoniae types A and B were cultured for 16 hours at 37°C on meat infusion agar and the growth emulsified in 0.9% saline containing 0.3% formalin. The same suspensions were used throughout the course of injections since the organisms retained their capsules unimpaired over several months on storage at 4-5°C. For injection the desired quantity of stock suspension, at a density of approximately 5 x 10^9 bacilli per ml, was diluted in saline to 2-5 ml.

H. influenzae type B (strain 62B) was subcultured weekly in fresh rabbit blood at 37°C for 16 hours; between passages the infected blood was stored at 4-5°C. To prepare antigen, the stock blood culture was streaked out on infusion agar containing 5% of Fildes' peptic digest of blood. After incubation overnight, the culture was checked microscopically for purity and re-streaked on a warm plate of Fildes' agar which was incubated for 6 hours at 37°C. The resulting growth was removed with a sterile cotton swab, emulsified in 0.9% saline, diluted to a turbidity corresponding to approximately 7-8 x 10^8 cells per ml (direct microscopic count) and the desired amount of this standardized suspension was further diluted to 2 ml with buffered saline pH 7.4. The suspensions were freshly prepared before each injection and checked for encapsulation by Quellung test.

Production of Antisera. Adult chickens of various breeds, weighing 5-7 lb, were used. They were injected in a wing vein and small bleedings were also taken from such veins. In the rabbits, weighing 5.6-6 lb, injections and small bleedings were made via an ear vein. In order to follow the rate of development of antibodies in the various animals

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TABLE I.
Production of Anti-*K. pneumoniae* Quellung Serum in Chickens.

Bird No.	Immunization schedule: Antigen employed	No. of injections	No. of bacteria per injection	Duration of course of injections	Interval between last injection and bleeding	Serum Quellung titer
	type		billions	days	days	
70	A	6	1.0-5.0	16	7	1:2
76	A	4	0.5-1.0	21	7	1:4
74	B	4	0.5-1.0	94	8	1:4
75	B	4	0.5-1.0	21	7	1:16

trial bleedings were made periodically, usually just prior to an injection of antigen. For large bleedings cardiac puncture was employed without anaesthesia; 30-50 ml was withdrawn without obvious harm to the animals of either species and on exsanguination up to 150 ml of blood was obtained from the birds. The blood was defibrinated with glass beads or allowed to clot spontaneously; serum was removed and stored with 0.01% merthiolate as preservative.

Capsular swelling tests. For *K. pneumoniae* the same stock suspensions of types A and B organisms used for the injection of animals were satisfactory for Quellung. For *H. influenzae* type B, 6-hour culture suspensions were freshly prepared and kept at 0°C during the period required to make the microscopic mounts. In each case the suspensions were adjusted so that the final mixtures would show 1-5 organisms per oil immersion field. The ability of the organisms to show Quellung was checked with commercial rabbit antiserum. Serial doubling dilutions of antiserum in saline were prepared; 2 loopfuls of a given dilution were mixed on a coverslip with a loopful of Loeffler's methylene blue solution and a loopful of bacterial suspension. Microscopic examinations of the moist preparations were made after the mixtures had stood at room temperature for 15 and for 30 minutes. For *H. influenzae* the various bleedings of the several animals were compared at the same time and the results checked by two competent observers.

Anti-K. pneumoniae serum. In Table I are summarized data on the production of antisera versus *K. pneumoniae* types A and B in roosters. In the first animal of the series, No. 70, 6 injections were made at intervals of 2-5 days and the dosage of organisms was higher than that employed for the later birds; serum collected 23 days after beginning the experiment gave excellent capsular swelling of the homologous antigen at dilution of 1:2. Bird 74 received 2 injections of *K. pneumoniae* type B suspension at an interval of 4 days; it was left for 81 days and then given a third dose. Five days later the animal was bled and the undiluted serum was found to exhibit Quellung. A fourth dose of antigen was administered 9 days after the third injection and the serum of the subsequent bleeding showed capsular swelling demonstrable at a dilution of 1:4. Roosters 75 and 76 each received 4 injections at weekly intervals and were bled 7 days after the last dose. The serum of bird 75 showed good capsular swelling versus type B organisms at dilutions as high as 1:16; rooster 76 serum was similarly active at 1:4 dilution versus type A capsular antigen.

Anti-H. influenzae serum. In this experiment an attempt was made to compare the

TABLE II.
Potency of Anti-*H. influenzae* Type B Serum Produced in Chickens and Rabbits.

Animal: Species	Sex	No.	Serum Quellung titer*
Chicken	♂	77	1:8
,,	,,	78	,,
,,	,,	81	,,
,,	♀	73	,,
,,	,,	80	,,
Rabbit	♂	315	undiluted
,,	,,	317	,,
,,	♀	314	,,

* Titer determined on bleeding taken 7 days after the fourth injection.

antibody response of chickens and rabbits when given the same quantities of *H. influenzae* type B, using suspensions of living organisms from young cultures. Each animal was given an initial dose of approximately 4 x 10^8 organisms followed by 3 or 4 injections of 8 x 10^8 cells each, at intervals of 7-8 days. The injections were well supported by the chickens, which showed no signs of illness. Among the 5 rabbits which were started in the experiment, 2 died following the first injection. The heart blood of one which was cultured at autopsy yielded a luxuriant pure growth of *H. influenzae*. It was not possible to autopsy the second rabbit promptly, so it was discarded.

One week after the second injection, 14 days after starting the immunization, 2 of the 3 chickens examined had already produced enough antibody to give definite capsular swelling with their undiluted serum. One week after the third injection, 20 or 22 days from the start, the serum of all 5 birds showed good Quellung at dilutions up to 1:8; no change in titer was observed after the fourth injection nor following a fifth injection made 8 to 10 days after the fourth, in the sera of 4 birds examined 8 to 12 days later.

By contrast, in the 3 rabbits which received the same course of injections, bleedings made after the fourth injection showed only a suggestion of capsular swelling even with undiluted serum and in the 2 animals from which a bleeding was obtained 8 days after the fifth injection, no increase in titer was noted.

Discussion. The notion that for Quellung tests rabbit antisera are inherently superior to those prepared in other species of animals has been shown to be erroneous. The determining factor is the quantity of anti-capsular antibody contained in the serum and equine antisera of comparable potency give capsular swelling as readily as those from rabbits(4,13). Our studies indicate that chickens likewise are eminently suitable animals for the production of capsular swelling antisera. While effective antisera have been obtained following various immunization schedules, we have found a procedure involving 3 or 4 injections separated by weekly intervals to be both rapid and convenient.

As regards the stability of chicken antibodies on prolonged storage, anti-meningococcal sera(12) preserved at 4-5°C have been tested 3½-4 years after their production and were found to have retained undiminished type-specific Quellung activity. Although we have not yet had opportunity to conduct tests with the anti-*H. influenzae* and anti-*K. pneumoniae* sera after similar periods of storage, our evidence to date indicates potency is maintained for at least a year.

Alexander(5), who introduced the capsular swelling test for the rapid diagnosis of meningitis due to *H. influenzae* type B, was able to produce rabbit antisera with an average Quellung titer of 1:10 by administering to her animals formalinized suspensions from 6-hour cultures in 3 series of 6 daily injections each with intervening rest periods of 8 days. The titer could be raised to 1:320 by prolonging the course of immunization to 24 weeks. In our experiment, antibodies adequate in concentration to give marked capsular swelling at serum dilutions up to 1:8 were regularly obtained in chickens after 3 or 4 injections of antigen, at a time when the corresponding rabbit sera were ineffective. The apparent superiority of chickens over rabbits for the production of antibodies reacting in other ways has been suggested by several investigators, among them Wolfe and Dilks(14,15) who emphasized the rapidity and regularity with which precipitating antibodies develop in chickens providing the latter are over 5 weeks old. Hilleman(16) has also pointed out the advantages of using chickens rather than rabbits for the production of neutralizing antibodies against the lymphogranuloma-psittacosis group of viral agents.

In addition to the ease and rapidity of production of antibodies in the chicken, the apparent innocuousness of *H. influenzae* for this species as compared with rabbits is note-

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worthy. In experiments directed toward the preparation of therapeutic anti-*H. influenzae* serum, Alexander and Heidelberger (17) found it necessary to extend the hyper-immunization over several months; they gave their rabbits multiple courses of injections each consisting of 4 daily doses of vaccine weekly for 4 weeks. A similar schedule was employed by Eaton *et al.* (18) for large-scale commercial production of antiserum. Both groups of workers noted (17,19) an appreciable mortality rate among the rabbits undergoing immunization. While some of the deaths were undoubtedly the result of the bleeding procedure and others were presumably due to intercurrent infections or other adverse conditions in the animal colonies, it is not unlikely that a portion of the deaths were due to the toxicity of the vaccines, since Dubos (20) has shown that these organisms may produce lethal soluble substance *in vitro*.

Our limited data on the preparation of anti-*K. pneumoniae* sera in chickens confirm those obtained for *N. meningitidis* and *H. influenzae*. It is highly likely that similar antisera can be readily prepared for other antigenic types of *Klebsiella pneumoniae* and *H. influenzae*, as well as various other encapsulated organisms. The successful results to date have been obtained with organisms the type-specific capsular antigens of which appear to be predominantly carbohydrate in nature. Whether Quellung would be demonstrable with non-carbohydrate capsular antigens is at the moment problematical.

Summary and conclusions. 1. Antisera satisfactory in the Quellung reaction were prepared for *K. pneumoniae* types A and B by injection of adult chickens with formalinized suspensions of the encapsulated bacilli. As few as 4 injections at weekly intervals were sufficient for this purpose.

2. Effective antiserum was similarly prepared for *H. influenzae* type B. Capsular swelling was readily demonstrable with sera collected from chickens after 3 or 4 weekly injections of living organisms from young cultures, while sera from rabbits undergoing the same immunization schedule were distinctly inferior at this stage.

3. Chickens are very useful for the production of Quellung antisera, with particular advantages where Gram-negative bacteria are concerned.

The assistance of Mrs. Frances Fuller during the early part of the work is appreciated.

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Effects of Phosphorus^{32} on the Hamster. (18029)

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Phosphorus^{32} was used in therapy as early as 1936. Later, Lawrence, Scott and Tuttle (1) employed it in a study of chronic leukemia and recommended its clinical use. Erf (2) attested its use in treatment of polycythemia. At the present time it is the treatment of choice for many cases of polycythemia. Dosages of phosphorus^{32} sufficient to bring

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about complete symptomatic relief or remission of polycythemia may conceivably produce histological damage to sensitive tissue. The Histological Group of the Metallurgical Laboratory (Plutonium Project) at the University of Chicago reported considerable damage to the testes of mice treated intraperitoneally with 2.5μc of phosphorus^{32} per g of body weight(3).

Results of a preliminary study of the effects of phosphorus^{32} on the hamster are presented in this paper. The hamster was chosen as the test animal because of its possible use in future histological and radiogenetic research. Since there is little, if any, information available in the literature on the physiological and histological effect of irradiation on the hamster, an attempt was made to ascertain the toxicity of phosphorus^{32} for the hamster and its effect on the histology of the testis.

Experimental. Ten hamsters from 3 to 5 months of age were used. They were descendants of a pair of the Bear Strain, obtained from the General Biological Supply House. They were fed Purina Hamster Chow supplemented with greens, carrots and potatoes. The animals were not crowded and were in a healthy condition at the beginning of the experiment. Two of the 10 animals were used as controls. The remaining animals, in pairs, were injected intraperitoneally respectively with 1, 6, 8 and 10 μc of phosphorus^{32} (in the form of disodium phosphate) per gram of body weight. Animals treated with 1 and 6 μc/g were sacrificed after 24 hours and 30 days, those treated with 8 μc/g were sacrificed after 30 days, while those treated with 10 μc/g succumbed to the treatment in 11 days. The testes were taken intact from the animals with epididymis attached and fixed with Zenkers fluid overnight, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Tissue for autoradiographs was fixed overnight in 95% alcohol.

Damage to the testis from 1 μc of intraperitoneally injected phosphorus^{32} per g of body weight was questionable at 24 hours. At 30 days there were occasional seminiferous tubules which consisted only of a basement membrane and Sertoli cells. Destroyed cells and apparently undamaged cells of all types were observed in almost all tubules. No alteration of the interstitial and Sertoli cells was noted. Autoradiographs showed a pale gray shadow throughout the entire organ 24 hours after treatment. Damage to the testis 24 hours after intraperitoneal injection of 6 μc of phosphorus^{32} per g resembled that found 30 days after injection of 1 μc. However, a larger number of damaged tubules was observed. Thirty days after treatment with the larger dosage a marked decrease in the size of the testis was noted. Tubules were completely void of spermatogenic cells, but contained Sertoli cells and a loose protoplasmic network (Fig. 1, A). Roughly one per cent of the tubules contained sperm. Thickening of the basement membrane was prominent. Interstitial tissue appeared aggregated making it difficult to distinguish it from connective tissue. It is possible that some of the interstitial cells were affected. Damage to the testis 30 days after treatment with 8 μc of phosphorus per g was similar to that resulting from injection of 6 μc insofar as could be observed by histological examination, except that the protoplasmic network was lacking in the lumen of many of the tubules (Fig. 1, B). Damage to the testis 11 days after treatment with 10 μc/g of phosphorus^{32} was questionable.

The general condition of the animals treated with 8 and 10 μc/g of body weight contributed information regarding the toxic effect of phosphorus^{32} on the hamster. Both groups of animals showed radiotoxic symptoms indicated by loss of appetite, loss of weight and inflammation of eyes. Inflammation of eyes began 5 days after treatment and persisted through the eighth day. Recovery was evident in animals treated with 8 μc/g about the ninth day. By this time, eyes which had been closed since the sixth day were opening. Ten days after injection all symptomatic signs had disappeared. Animals treated with 10 μc/g showed no sign of recovery and both died on the eleventh day.

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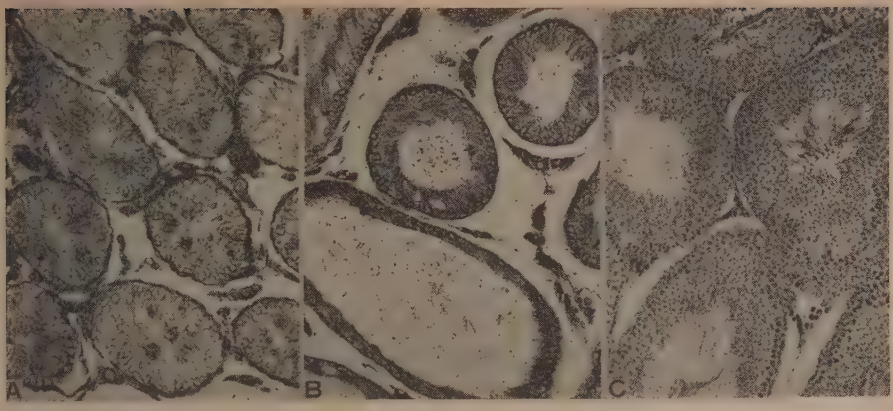

FIG. 1.

Sections through testes of 4-months-old hamsters. (A) 30 days after injection with 6 μc/g of phosphorus^{32}, (B) 30 days after injection with 8 μc/g of phosphorus^{32}, and (C) healthy controls. Note complete absence of spermatogenesis in both A and B.

The higher dosages of phosphorus^{32} (6 and 8 μc/g) proved extremely destructive to the testis. Depopulation was complete in 30 days and there was no evidence of regeneration at this time. While the physiological and histological effects of irradiation are known to be dependent upon the time of exposure, it seems probable that a period of 30 days is sufficient for development of the maximum effects even with the lowest dosage of phosphorus^{32} used. According to Schinz and Slotopolsky(4), regeneration of spermatogenic cells, if it takes place at all, begins before height of depopulation is reached. It is claimed by others(5) however, that restitution may begin 4 or more months after depopulation. Thus, although the results suggest that injection of 6 or more μc of phosphorus^{32} per g is sufficient for permanent sterilization of the hamster, further work is needed to establish the exact dosage required.

Summary. Effects of intraperitoneal injection of the hamster with phosphorus^{32} are described. Animals treated with 10 μc/g of body weight died in 11 days, there were no apparent changes in the testis at this time. Toxic effects were observed in animals injected with as little as 6 μc/g. Damage to the testes of animals treated with 6 and 8 μc respectively, was extensive, consisting of marked decrease in size, absence of spermatogenic cells in tubules, and actual damage to the tubules themselves, suggesting permanent sterility. No changes in the testes of animals treated with 1 μc/g were apparent in 24 hours. However, some damage was observed 30 days after treatment with 1 μc. Spermatogenic cells were more sensitive than Sertoli cells. Some interstitial cells appeared to be affected.

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Agglutination of Bacteria by Lymphoid Cells *in Vitro*. (18030)

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While some investigators still believe that the lymphocytes are cellular sources of antibodies(1), others have come to the conclusion that it is the plasma cell rather than the lymphocyte which elaborates these globulins (2-5). As there has been no direct evidence for either theory, we have continued to search for a crucial experiment. We believe that this has been found. It was observed that certain lymphoid cells of antibody forming lymph nodes exhibited on their surface an antigen-antibody reaction when exposed to antigen *in vitro*. Polymorphonuclear leucocytes, monocytes and macrophages failed to show this phenomenon.

Method. Antibody production was induced in the popliteal lymph nodes of rabbits through injection into the footpads of 0.5 ml of a commercial typhoid H or brucella antigen ("febrile antigen," Lederle). After intervals varying from 2 to 14 days, the nodes were removed and their cells suspended in physiological saline solution. The antibody contained in the lymph plasma was washed out by twice repeated centrifugation and resuspension in saline. Of the final suspension, a drop was placed on a slide and covered with a drop of a similarly washed and suitably diluted portion of the antigen. The preparation was then sealed and studied microscopically.

Results. During the first 10 to 15 minutes following the addition of bacteria to the cells, both the bacteria and the cells were evenly distributed throughout the microscopic fields. Thereafter some bacteria began to stick to the surface of certain lymphoid cells. After 30 to 45 minutes this phenomenon was at its peak. The agglutination of the bacteria on the surface of the cells was associated with loss of Brownian movement.

The number of cells showing this phenomenon was greatest 5 to 7 days following the injection of antigen. It was smallest on the third and fourteenth days, and there was none on the second. The percentage of cells showing the phenomenon was not determined. During the height of the reaction we often found 2 to 3 such cells in one microscopic field.

The nature of the agglutinating cells was difficult to determine when unstained or supravitally stained preparations were used. The addition of Giemsa, Wright or some other stains caused separation of cells and bacteria. However, when the drops were placed on dried films of an alcoholic Janus Green solution, satisfactory results were obtained. Fig. 1 and 2 were made from unstained preparations, all others from preparations with Janus Green.

Fig. 1, 4, 6, 7, 9, 10 and 11 leave no doubt that many of the agglutinating cells were plasma cells. However, only a certain number of the identifiable plasma cells showed this phenomenon. Fully mature plasma cells were more often negative than positive.

Other agglutinating cells had the appearance of immature lymphoid cells (Fig. 2, 3, 4, and 5). Many of these were obviously plasmablasts as shown by the coarseness of their mitochondria and the abundance and deep basophilia of their cytoplasm. These immature elements agglutinated bacteria more often and more strongly than the mature cells indicating a loss of this function during maturation (Fig. 4). Immature lymphocytes which were present in large numbers during

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the second week of the experiment were not observed to agglutinate.

The cell which seemed to exhibit the phenomenon most often when present was comparatively small, though larger than a small lymphocyte (Fig. 11). It had a round, rather diffusely stained nucleus and usually a good deal of cytoplasm. Because it was so poorly differentiated structurally, it is felt that this cell was close to mitotic division either arising from it or about to divide. The nature of this cell could not be established. In view of all other findings, however, it may be assumed that it belonged to the plasma cell series.

Typical small lymphocytes never showed the phenomenon (Fig. 1 to 8). The only agglutinating cell found in our preparations that resembled a small lymphocyte is illustrated in Fig. 8. It can be seen that this cell is somewhat larger than a typical small lymphocyte. It also contains much more cytoplasm. It may well be that this was a plasma cell which had lost some cytoplasm as the result of the centrifugation or for some other reason. That plasma cells through loss of cytoplasm may approach the appearance of lymphocytes can be seen in Fig. 12.

Discussion. It has thus been demonstrated that certain lymphoid cells of antibody forming lymph nodes exhibit agglutination on their surface when exposed to antigen *in vitro*. That this was not an artefact, but true agglutination is apparent from the facts that it occurred only between cells of immunized rabbits and the bacteria with which this immunization was achieved, that it was at its peak when the antibody concentration in the lymph nodes was highest, and that there were no cross reactions when typhoid antigen was added to cells from rabbits immunized with brucella organisms and vice versa.

Many of the agglutinating cells were identified as plasma cells or their predecessors. Polymorphonuclear leukocytes, monocytes and macrophages as well as typical small lymphocytes were ineffective. The observation that immature plasma cells agglutinated better than mature elements, and cells which were interpreted as close to mitotic division were particularly effective, could be construed into a confirmation of the theory of Fagraeus(3) that the production and release of antibody by these cells was a function of their youth. It is equally conceivable, however, that the loss of agglutinating power of the more mature cells was due to maturation of the cell membrane. It may be assumed that the regenerating cuticula after mitotic division should at first be delicate enough to allow contact between antibody within the cell and antigen without, whereas in the mature cells it may be so thick as to prevent antigen-antibody union.

Experiments with cortisone and similar substances* which are believed to cause shedding of cytoplasm(6) did not distinctly enhance the phenomenon. Cortisone in doses of 50 γ per ml caused considerable acceleration of intracellular vacuolization especially in the plasma cells and budding of cytoplasm especially in the lymphocytes, but these phenomena occurred also in the absence of such substances though less rapidly, and therefore are interpreted as agonal phenomena. As this acceleration was effected only with large doses of cortisone, we suspect that it was due to the solvent contained in the commercial preparation rather than the hormone. It is interesting that the plasma cells differed from the lymphocytes by less budding. This is in keeping with our impression that mature plasma cells have a thicker cell membrane than small lymphocytes.

Testicular hyaluronidase, desoxycorticosterone acetate and Artisone (21-acetoxypregnenolone) in doses up to 125 γ per ml had no appreciable effect on the cells or the agglutination phenomenon.

Summary. It has been demonstrated that certain lymphoid cells of antibody forming lymph nodes agglutinate on their surface *in vitro* the bacteria with which the animals were immunized. The agglutinating cells which could be identified belonged to the

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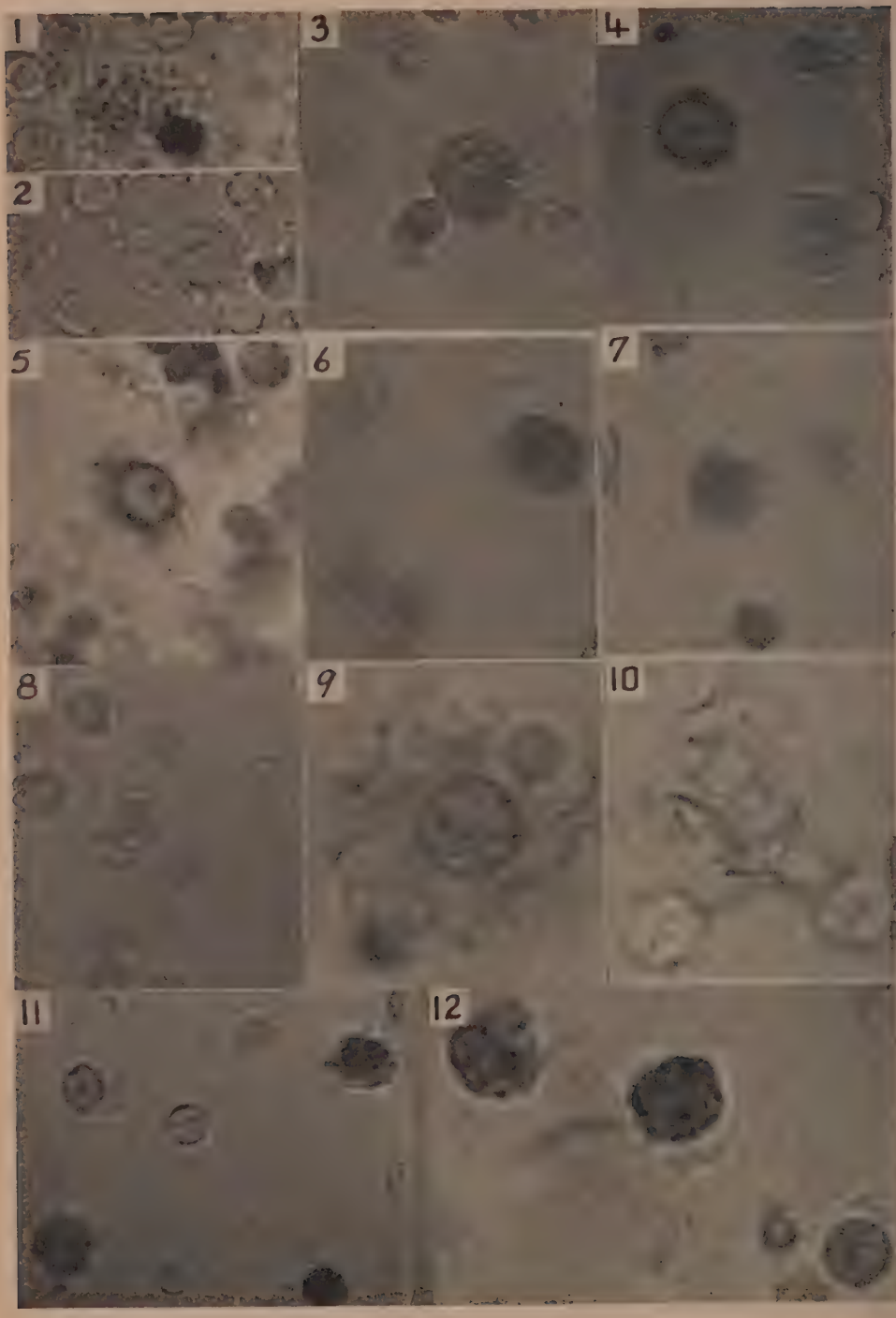
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Fig. 1 to 4 and 11 show agglutination of brucella, Fig. 5 to 10 that of typhoid H. The large rods in Fig. 12 are Cortisone rods. Fig. 1 and 2 were made from unstained preparations, all others from preparations stained with Janus Green. Fig. 9, 10, and 12 were magnified × 2000, all others × 1000.

plasma cell series, whereas typical small lymphocytes failed to show the phenomenon. These observations seem to show that it is the plasma cell rather than the lymphocyte which elaborates agglutinins.

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Prevention of Chemotherapeutic Effects of 4-Amino-N^{10}-Methyl-Pteroylglutamic Acid on Mouse Leukemia by Citrovorum Factor.* (18031)

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4-Amino-N^{10}-methyl-pteroylglutamic acid has previously been shown to be effective in prolonging the survival time of mice with transplanted leukemia AK4(1). This prolongation of survival time can be prevented by prior administration of massive doses of pteroylglutamic acid (PGA) or pteroyltriglutamic acid (PTGA)(2). Since the citrovorum factor (C. F.)(3) has been shown to be more effective than PGA in preventing the toxicity of 4-amino-PGA in the bacterium, *Leuconostoc citrovorum*(4), and in mice(5) attempts were made to prevent the antileukemic effect of 4-amino-N^{10}-methyl-PGA by C. F. (6). The detailed results of these studies are herewith reported.

Method. Experimental.—The procedure for inoculating mice of the AKm stock with AK4 leukemia was similar to that reported previously(2). 0.1 cc of a saline suspension of leukemic spleen containing 1,000,000 cells was injected intraperitoneally. Forty-eight hours later, treatment with 4-amino-N^{10}-methyl-PGA was started at a dose of 2 mg/kg three times weekly for ten doses by the intraperitoneal route. Intraperitoneal injections of citrovorum factor were started twenty-four hours after the inoculation of the leukemia and then given fifteen minutes before each dose of the antimetabolite. Various dosage levels in units(3) of C. F. were studied against the standard dose of 4-amino-N^{10}-methyl-PGA. In one experiment various levels of C. F. and PGA were given one hour after each injection of 4-amino-N^{10}-methyl-PGA. In another experiment the C. F. was allowed to stand at pH 2.0 for 24 hours at room temperature before injection in order to destroy the C. F. activity(5). After this procedure all the folic acid activity but only 10% of the C. F. activity remained as shown by microbiological assay against *S. fecalis* and *L. citrovorum* respectively.

Discussion. The preparations of citrovorum

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TABLE I.
Prevention of Chemotherapeutic Effect of 4-Amino-N^{10}-methyl-PGA by C.F. and PGA.

Exp.	4-amino-N^{10}-methyl-PGA in mg/kg	C.F. in u/kg × 10^3	PGA in mg/kg	No. of mice	Range of survival time, days	Mean survival time, days
1	—	—	—	10	9-14	11.6
	2	—	—	10	21-35	27.6
	2	5000*	—	10	11-15	13.5
	2	2500*	—	10	11-15	13.1
	2	1250*	—	10	14-19	15.5
	2	625*	—	10	15-28	18.6
	2	—	7.5†	9	15-35	23
	2	—	3.7†	8	14-32	25
	2	—	1.8†	10	19-35	27.7
2	—	—	—	10	7-11	9.0
	2	—	—	6	22-28	25.3
	—	5000	—	10	7-12	8.1
	2	5000*	—	10	7-19	11.4
	2	2500*	—	10	11-17	14.2
	2	1250*	—	7	10-16	12.3
	2	625*	—	10	14-21	16.2
	2	312.5*	—	10	10-22	17.5
	2	78.1*	—	10	16-35	20.7
	2	39.05*	—	9	16-28	21.1
	2	—	15*	9	11-16	13.7
3	—	—	—	30	7-15	8.8
	2	—	—	23	21-28	24.3
	—	3000	—	11	7-10	7.7
	2	3000‡	—	10	14-22	16.1
	2	1000‡	—	9	13-28	19.4
	2	300‡	—	8	11-30	21.9
	2	100‡	—	10	14-30	22.8
	2	—	30‡	6	20-28	23.0
	2	—	10‡	9	20-29	25.2
	2	—	3‡	11	11-28	22.2
4	—	—	—	9	7-13	9.2
	2	—	—	10	15-27	22.8
	2	1250*	—	6	14-20	15.2
	2	2500* (acid hydrolyzed)	—	10	14-27	21.4
	2	1250* (acid hydrolyzed)	—	10	20-27	24.5
	2	625* (acid hydrolyzed)	—	8	20-27	23.1

* Injected ¼ hr before the injection of 4-amino-N^{10}-methyl PGA.
† Injected 1 hr before 4-amino-N^{10}-methyl PGA.
‡ Injected 1 hr after 4-amino-N^{10}-methyl PGA.

factor used in these experiments had an activity for *L. citrovorum* of 3,000,000 units(3) per cc and contained 3 mg/cc of organic material. Thus, this impure preparation of C. F. can be considered as having 1,000,000 units of activity per mg.

As can be seen from Table I, 625,000 units/kg to 1,250,000 units/kg (0.62 to 1.25 mg/kg) of C. F. are approximately as active as 15 mg/kg of PGA in preventing the antileukemic effect of 4-amino-N^{10}-methyl-PGA, and on a dry weight basis C. F. is thus 12 to 24 times as active as PGA. When PGA or C. F. were given one hour after the antagonist, 30 mg/kg of the former was without effect, but C. F. showed some effect at 3 mg/kg of dry material.

When the preparation of C. F. was allowed to stand at room temperature for 24 hours at pH 2.0, which did not diminish its folic acid activity, its effectiveness in preventing the effects of 4-amino-N^{10}-methyl-PGA was markedly decreased, further emphasizing that this effect of the preparation of C. F. was due

to its C. F. content and not to free folic acid possibly present as a contaminant or formed by the action of the hydrogen ion on the C. F. preparation.

Since 1 cc of the C. F. preparation containing 3.0 million C. F. units had a folic acid activity equivalent to approximately 1.0 mg of PGA as determined by microbiological assay with *S. fecalis*, 0.42 cc of the preparation, containing 1.25 million units, had a folic acid activity corresponding to 0.42 mg of PGA. Thus the preparation was 36 to 72 times as potent in preventing the antileukemic effects of 4-amino-N^{10}-methyl-PGA as could be expected from its total folic acid activity.

Summary. The effect of 4-amino-N^{10}-methyl-PGA in prolonging the survival time of mice with transplanted leukemia AK4 can be blocked almost completely by prior administration of 1/3 to 2/3 as much by dry weight of a preparation of citrovorum factor. Thus, citrovorum factor is at least 12 to 24 times as active as pteroylglutamic acid in preventing the antileukemic effect of 4-amino-N^{10}-methyl-PGA.

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Pressure Gradients in the Atria and Pulmonary Veins in Man. (18032)

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Careful analysis of the pressure records obtained from the pulmonary veins and chambers of the heart was carried out on 4 noncyanotic persons selected because of their varying anatomic and physiologic defects. In each instance, the pressure in the pulmonary vein was found to be higher than pressures measured in either or both atria. The cases form a unique group: in 2 the pulmonary vein, being anomalous, drained into the superior vena cava and the right atrium respectively; in 1 there was an atrial septal defect with a large left-to-right shunt; and in the fourth there was a patent foramen ovale, without demonstrable shunt, associated with moderate pulmonary stenosis. The observations on the pressure gradients are in accord with the early reports of Cournand and associates(1) and of Dexter and associates(2,3), who reported their findings in patients with atrial septal defect. One of the purposes of this study was to investigate, by detailed examination of pressure pulses, the concept that the gradient of pressure producing flow of blood from left to right atrium results from pressure transmitted through the pulmonary circulation by the right ventricle. This view was not expressed by Little and associates(4).

Methods. Records of the 4 patients adequate for analysis were obtained over a short sequence of time from various cardiac chambers. In each instance the tip of the cardiac catheter entered a pulmonary vein and was confirmed in a position several centimeters distal from the atrial opening by roentgenography. Recorded pressure and oxygen content of a sample of blood were also measured with the catheter tip in the same location.

Simultaneous records of blood pressure, of respiratory cycle and of electrocardiogram were made in each instance except in Case 1, in which respiration was not recorded. For measurement of pressure a strain gauge manometer was attached to the external end of the catheter. The reference point of zero pressure was located at the midpoint of the thorax at the level of the third rib at the

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TABLE I.
Pressures in Millimeters of Mercury Measured at Indicated Points in One Cardiac Cycle, Through Catheter in Pulmonary Vein (P.V.), Left Atrium (L.A.), and Right Atrium (R.A.).

	Case 1			Case 2 Phase of respiration			Case 3		Case 4	
	Midinspiration			End expiration			End inspiration		Midexpiration	
Phase of cardiac cycle	P.V.	L.A.	R.A.	P.V.	L.A.	R.A.	P.V.	R.A.	P.V.	R.A.
Onset of P wave	10.3	5.9	6.7	6.9	2.6	1.3	3.3	—2.6	9.2	3.7
0.1 sec. before R wave										
Mid P-R interval	6.8	7.4	8.2	3.8	0	1.7	3.2	—0.3	10.0	5.6
Onset of R wave	13.1	8.9	9.3	3.7	4.1	0.7	4.7	1.4	10.7	8.8
Mid R-T interval	10.3	3.9	3.6	1.2	0	—0.4	2.4	—2.9	14.8	5.6
End of T wave	10.8	10.1	6.8	5.3	1.9	1.4	3.5	0		
Midpoint of electrical diastole	10.4	8.4	9.2	7.0	2.0	1.9	6.2	1.4	16.1	8.2
0.1 sec. after T wave	—	—	—	—	—	—	—	—	—	—
Avg	10.3	7.4	7.3	4.6	1.8	1.1	3.9	—0.5	12.2	6.4

sternal border.

In order to achieve comparable data for pressures which were not measured simultaneously, single cardiac cycles of reasonably equal length and occurring in the same period of the respiratory cycle were selected for detailed measurement from the photographic record made in each cardiac chamber and great vessel. In Case 1, wherein a record of respiration was not made, comparable cardiac cycles were chosen from the record on the basis of pressure relationships noted on those records in which respiratory tracings were present. Measurements of the pressure recordings were made at the following phases of the cardiac cycle: onset of P wave, mid P-R interval, onset of R wave, midpoint of R-T interval, end of T wave, and midpoint of electrical diastole, except in Case 4 in which auricular fibrillation was present and measurements could not be made at the first two sites (Table I). An example of the type of record from which this study was made appears in Fig. 1. These pressure tracings were taken from Case 2 wherein the catheter entered all 4 cavities of the heart.

In order to eliminate possible error due to failure to achieve comparable phases of respiration in selecting cardiac cycles, pressures were compared in Cases 2 and 3 both in end expiration and in end inspiration. In Case 4 pressures were also recorded in midexpiration and in midinspiration. The gradients of pressure were found to be consistent in all cases.

Results. Catheterization of the heart in Case 1 yielded evidence for an atrial septal defect with left-to-right shunt. The average gradient between pulmonary vein in the lung and left atrium proved to be 2.9 mm of mercury, between pulmonary vein and right atrium 3.0 mm (Table I). The calculated flow through the pulmonary circulation was 15.3 liters per minute.

Catheterization of the heart in Case 2 revealed a patent foramen ovale without demonstrable shunt (no arterialization of blood in the right atrium). A ventricular septal defect and mild pulmonary stenosis were also present. The average gradient between pulmonary vein within the lung and left atrium,

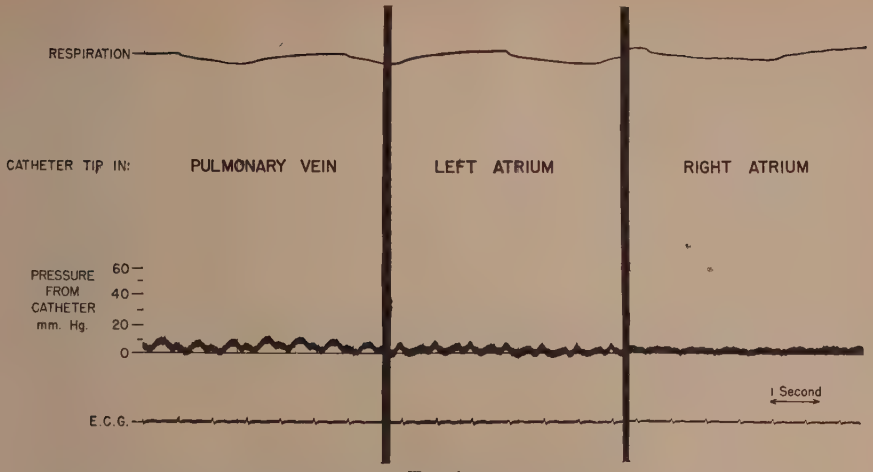

FIG. 1.
Low pressure tracings recorded through catheter with tip in sites indicated (Case 2).

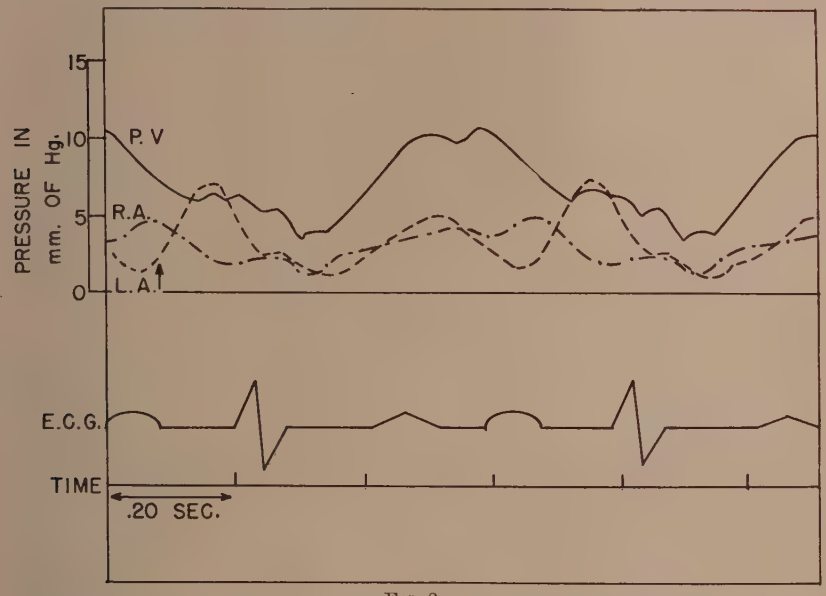

FIG. 2.
Superimposed tracings from fivefold photographic enlargements of the pulse contours recorded during one cardiac cycle in pulmonary vein (P.V.), right atrium (R.A.), and left atrium (L.A.) (Case 2). All cycles selected in midexpiration.

at the end of expiration, was in this case 2.8 mm of mercury, between pulmonary vein and right atrium 3.5 mm (Table I). The flow through the pulmonary circulation as measured by the direct Fick principle was 9.2 liters per minute.

In the catheterization procedure on Case 3 a right pulmonary vein was entered from the lower portion of the superior vena cava. The average gradient between pulmonary vein and right atrium was 4.4 mm of mercury (Table I). The calculated flow through the pulmonary circulation was 11.7 liters per minute.

Catheterization of the heart in Case 4 revealed an anomalous pulmonary vein draining into the upper part of the right atrium. The average pressure gradient between pulmonary vein and right atrium was 5.8 mm of mercury (Table I). The pulmonary flow was 12 liters per minute.

Comment. In the 4 cases studied, a pressure gradient between a pulmonary vein several centimeters distal to the atrial opening and either or both atria during the major portion of the cardiac cycle was demonstrated. This gradient is illustrated in Fig. 2, which consists of tracings selected at midexpiration from the actual records from pulmonary vein and atria in Case 2. The illustration was prepared by tracing fivefold photographic enlargements of the pulse contours during a cardiac cycle. The average pressure gradient between pulmonary vein and right atrium in the 4 cases studied was 4.2 mm of mercury. This value compares well with those previously reported in the literature(1,2,5).

Possible sources of error in measurement of pressure require comment in this study because of the relatively small differences observed. One likely source could result from differences in the position of the catheter tip relative to the arbitrarily selected zero point of midthoracic position. But error due to this factor does not seem likely to account for the consistent differences in pressures observed.

Of greater possible significance as a source of error is the position of the orifice of the catheter relative to the flow of blood. A model was built in order to investigate this end pressure effect. A No. 8 catheter was mounted in a glass tube 1 meter in length with an internal diameter of 1 cm, which was connected through rubber tubing to a water faucet. The pressures were recorded photographically by two 0.2 lb strain gauge manometers; one was connected to the catheter, the other to a T tube sealed perpendicularly into the side of the 1 cm glass tube near its midpoint. Simultaneous recordings of pressures were taken at different flow velocities. An analysis of the pressure tracings showed that a flow velocity of 80 cm per second directed against the open orifice of the catheter was required to produce a pressure 2.5 mm greater than the true lateral pressure. These data agree with those of Haddy and Visscher(6), who made a similar experiment using a No. 10 catheter.

In Case 1 of this series, in which there was the greatest amount of shunt, the pulmonary flow calculated by the direct Fick principle was 15.3 liters per minute. Assuming that this flow was distributed equally in four pulmonary veins and that the diameter of the pulmonary vein catheterized was 1.3 cm, the lower limit of values for the diameter of normal pulmonary veins measured by Charpy (7), the velocity of flow in this vessel would be 48 cm per second. According to the data obtained on the model built in this laboratory, a flow of this magnitude could account for a pressure only 0.8 mm of mercury greater than true lateral pressure. The observed difference in pressure in Case 1 between pulmonary vein and the left atrium, in which the position of the orifice with respect to flow was not clear, proved to be 2.9 mm of mercury. In Case 2, in which pulmonary flow was but 9.2 liters per minute, the lowest pulmonary flow of the 4 cases, the end pressure effect in a vein 1.3 cm in diameter could account for but 0.5 mm of mercury difference whereas the recorded difference in pressure between pulmonary vein and left atrium was 2.8 mm of mercury. It does not seem likely, therefore, that an end pressure effect was the only phenomenon being observed in the cases reported.

Furthermore, the pressures recorded from the venae cavae were significantly lower than those observed in the pulmonary veins, and there was a consistent difference in inflow pressure into the two atria averaging 6 mm of mercury in these cases. It must also be noted that the measurements taken with the present technics are in agreement with those

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6. Haddy, F. J., and Visscher, M. B., personal communication to the authors.

7. Charpy, A., Systéme Veineux, in Charpy, A., Nicolas, A., Prenand, A., Poirier, P., and Jacques, P., *Traité d'anatomie humaine*, Paris, Masson et Cie, 1898, vol. 2, p. 883.

of other authors using different methods. Hamilton and associates(8), measuring pressures with a needle directly inserted into the vessels of normal unanesthetized dogs breathing quietly, recorded in the pulmonary veins average pressures of 3 to 12 mm of mercury. In this study, average pressures recorded in the pulmonary veins were 3.9 to 12.2 mm.

A lesser distensibility of the left atrium cannot account for the high pressures observed in the pulmonary veins draining into vena cava and right atrium.

Summary. 1. During cardiac catheterization, a pressure gradient was found between a distal point in a pulmonary vein and either or both atrial chambers during the major portion of the cardiac cycle in 2 cases of atrial septal defect, in a third case wherein an anomalous pulmonary vein drained into the right atrium, and in a fourth in which an anomalous pulmonary vein emptied into the superior vena cava.

2. The magnitude of the gradient between pulmonary vein and *right* atrium varied from 3.0 to 5.8 mm of mercury with an average value of 4.2 mm in the 4 cases studied.

3. Evidence that this gradient could not be entirely due to an end pressure or Pitot effect was obtained from study of a model in which the end pressure effect was shown to be 0.5 to 0.8 mm of mercury at the same calculated flow velocities.

4. It is suggested that this pressure gradient is a result of the pressure transmitted by the right ventricle through the pulmonary circulation, and is an important factor in the left-to-right shunt observed at rest during quiet breathing in uncomplicated cases of atrial septal defect.

8. Hamilton, W. F., Woodbury, R. A., and Vogt, Elkin, *Am. J. Physiol.*, 1939, v125, 130.

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Effect of Choline Chloride on Development of Atherosclerosis in the Rabbit.* (18033)

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The demonstration of the lipotropic activity of choline has led to the testing in experimental animals of its ability to prevent the arterial lipid deposition characteristic of atherosclerosis. Although some observers(1,2) have failed to find any protective action, others(3-6) have claimed that choline administration retards the development of lesions.

In investigating this subject we have administered choline chloride to rabbits by force-feeding gelatin capsules rather than by mixing choline with the food, not only to ensure a known and uniform dosage, but also in order to avoid a possible depression in the food intake of choline-treated animals due to the brackish taste of the chloride. It has previously been shown(7) that caloric restriction with consequent weight loss or decreased rate of weight gain inhibits the development of atherosclerotic lesions.

Methods. Fourteen adult male rabbits of various breeds, kept in individual cages and allowed Purina Rabbit Chow Checkers and water *ad libitum*, were used as experimental

* This study was supported by a grant from the Canadian Life Insurance Officers Association.

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4. Kesten, H. D., and Silbowitz, R., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 71.

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TABLE I.
Summary of Experimental Data.

Animal	Avg blood total cholesterol (mg %)	Initial wt (kg)	Ratio of final to initial wt	Avg food consumption per kg body wt (g per day)	Degree of atherosclerosis
1	101	3.79	1.12	41	0
2*	109	3.28	1.15	37	0
3	117	3.29	1.25	46	0
4	124	3.03	.96	35	1
5*	140	3.40	.93	32	1
6*	149	3.25	.96	30	2
7	154	3.32	1.08	40	2
8	166	3.39	.96	32	3
9*	202	3.35	.87	27	3
10	235	2.78	1.03	38	2
11	238	3.56	.92	35	3
12	271	3.26	.91	25	4
13	320	3.72	.81	25	4
14*	329	2.99	.93	31	3
		Means and Standard Errors.			
Choline	185.8 ± 38.8	3.254 ± .071	0.968 ± .048	31.4 ± 1.6	1.80 ± .58
Control	191.8 ± 25.6	3.349 ± .106	1.004 ± .044	35.2 ± 2.4	2.11 ± .51

* Animals given choline.

animals. Each animal received 1 g of cholesterol 6 days a week enclosed in 2 size 00 gelatin capsules. Five animals received, in addition, 1 g of choline chloride 6 days a week in capsules. The 9 controls received 2 empty capsules 6 days a week. In using this method of administration, suggested by Pollak(8) for cholesterol feeding, some difficulty was encountered in getting the animals to swallow the capsules and several rabbits were lost from respiratory obstruction before a satisfactory technic was developed. Good results were obtained when the capsule being administered was gently introduced into the pharynx while the animal's head was displaced (not rotated) backwards. Each animal was weighed weekly, its food consumption recorded daily, and its blood total cholesterol level determined weekly on heparinized ear vein blood by the Schoenheimer-Sperry method(9). After 9 weeks of cholesterol and choline administration the animals were killed and the whole aortas removed and fixed in 10% formalin. The aortas were stained with Sudan IV and graded as unknowns into 5 arbitrary groups according to the area of intima involved by lesions.

Results and discussion. The data are summarized in Table I in order of increasing average blood cholesterol level. Animals given choline are indicated by asterisks. The blood cholesterol averages do not include determinations made before the beginning of cholesterol administration. Food consumption values are averages of the 9 weekly averages computed for each animal on the basis of its average daily food intake and average body weight each week.

There is an obvious increase in the degree of atherosclerosis with increasing average blood cholesterol level. The two rabbits (10 and 14) whose degrees of atherosclerosis are less than would be expected from consideration of blood cholesterol levels alone had the lowest initial weights of the group. This is consistent with our previous finding(7) that animals of low initial weight tend to develop less atherosclerosis than animals of high initial weight, other factors being equal. However, with the foregoing exceptions, the variations in initial weight, weight change, and food consumption in the present experiment are relatively small and are insufficient to affect appreciably the correlation between degree of disease and blood cholesterol level.

8. Pollak, O. J., *Arch. Path.*, 1944, v37, 337.

9. Sperry, W. M., *The Schoenheimer-Sperry Method for the Determination of Cholesterol*, New York, New York State Psychiatric Institute and Hospital, 1945.

The means of the average blood cholesterol levels, initial weights, ratios of final to initial weight, food consumptions, and degrees of atherosclerosis in the choline-treated and control groups do not differ significantly.

The statistical significance of the difference between the mean degrees of atherosclerosis in the two groups adjusted to the common mean blood cholesterol level can be tested by the analysis of covariance. The adjusted means do not differ significantly, since the variance ratio F (0.23) is well short of the 5% point (4.84). Moreover, it is unlikely that any feasible increase in the number of experimental animals would lead to the demonstration of a significant difference under these experimental conditions; for it can be estimated that control and test groups of approximately 110 animals *each* would be required to demonstrate significance at the 5% level assuming that the relative standard deviation will be the same for the larger sample(10).

In a recent investigation(6), the results of which indicated that choline retarded the development of lesions, Steiner offered 1 g of cholesterol mixed with the food 3 times weekly to 54 rabbits. Ten were offered 1 g and 19 were offered 0.5 g of choline chloride daily mixed with the food. The remaining 25 served as controls. Assuming that choline was offered 6 days a week, and, assuming that equal proportions of the cholesterol and choline offered were actually ingested, the ratio of the cholesterol and choline weekly dosages in the 19 animals offered 0.5 g of choline daily was $3/3 = 1$. In our experiment, the ratio of cholesterol and choline weekly dosages was $6/6 = 1$, so that we believe our choline dosage relative to cholesterol dosage was comparable to that used by Steiner in his animals on the lower choline dosage.

A larger dosage of choline than we have used may inhibit the atherosclerotic process. However, in view of earlier findings(7), we believe that, in order to demonstrate that any agent specifically inhibits the development of lesions independently of any effect on blood cholesterol level or body weight, it should be shown that the treated and control groups do not differ significantly in duration or level of cholesterol administration, in average blood cholesterol level, in initial weight, or in relative weight change during the experiment.

Summary. The degree of aortic atherosclerosis found in 14 male rabbits given 1 g of cholesterol in gelatin capsules 6 days a week for 9 weeks showed a highly significant positive linear correlation with the average blood total cholesterol level.

Five of these animals which received 1 g of choline chloride in gelatin capsules 6 days a week for the 9 weeks did not differ significantly from the 9 control animals in average blood cholesterol level, in body weight, or in the degree of atherosclerosis which developed.

The author wishes to thank Dr. C. H. Best, under whose direction this work was performed, for his interest and helpful advice.

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Effect of Convulsant and Anticonvulsant Agents on the Activity of Cytochrome Oxidase.*† (18034)

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The cerebral oxygen consumption is known to be increased during convulsive seizures. Hence, an attempt was made to ascertain whether chemical agents known to induce convulsive seizures modify the activity of enzymes involved in oxygen consumption. The present report is a summary of the effects of convulsant and anticonvulsant agents on the activity of cytochrome oxidase.

Method. The effect of convulsant and anticonvulsant agents on the activity of cytochrome oxidase was investigated following the method of Schneider and Potter(1) as described by Umbreit and collaborators(2) consisting of measuring the oxygen uptake by an enzyme-substrate mixture. 0.3 ml of a $1x10^{-3}$M $AlCl_3$ solution, 0.3 ml of either water or a solution containing the convulsant and anticonvulsant agents in concentrations from $1x10^{-2}$ to $1x10^{-4}$M, either 0.5, or 0.75, or 1 ml of a suspension containing 0.2% tissue homogenate in water, and water in amounts to bring the total volume to 1.6 ml were added to the main chamber of a Warburg vessel. 0.1 ml of a 0.2 N sodium hydroxide solution with 1 sq cm folded filter paper was placed in the center cup. One ml of a 0.1M phosphate buffer at pH 7.4 containing cytochrome c in a concentration of $2.4x10^{-4}$M was added to one side arm of the Warburg vessel and 0.3 ml of a 0.114M sodium ascorbate solution at pH 7.4 was added to the other side arm. All water used was twice distilled in glass to avoid contamination with heavy metals. The solutions were ice-cold and were freshly prepared. During preparation the Warburg vessels were immersed in ice. Air was used as the gas phase for convenience. The water bath was kept at 37°C. After thermal equilibrium was reached the ascorbate and the cytochrome c were tipped from the side arms into the main chamber and the rate of oxygen uptake was measured. Readings were made every 10 minutes for half an hour. The oxygen uptake was approximately proportional to the time of incubation. The oxygen uptake in parallel runs differed less than 3%.

Preparation of the enzyme. Either rat liver or rat brain served as a source of the enzyme. Immediately before mounting the Warburg vessels containing the substrate mixture the animals were decapitated and a tissue aliquot was measured. The aliquot was homogenized by means of an apparatus described by Potter and Elvehjem(2,3) under ice, it was diluted with distilled water, and was added to the main chamber of the Warburg vessel. The endogenous oxygen uptake of the liver was insignificant.

Convulsant and anticonvulsant agents. The substances were used in low concentrations ($1x10^{-3}$M and less) at pH 7.4. This precaution was necessary to minimize changes in the buffering ability of the phosphate solution, a factor that modifies the activity of cytochrome oxidase by itself. Furthermore, many of the substances induced changes in the rate of autoxidation of the ascorbate if

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3. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, v114, 495.

used in final concentrations of $1x10^{-2}$M and more.

Autoxidation of the substrate. The rate of the autoxidation of the ascorbate was determined by extrapolation to zero tissue concentration from a series of 3 different tissue concentrations respectively(2). The autoxidation rate was obtained by subtracting the increments of oxygen uptake in the Warburg vessels containing 0.75 ml and 1 ml of homogenate from the oxygen uptake in the Warburg vessels containing 0.5 ml of the homogenate. The rate of oxygen uptake in 10 minutes averaged 5 μl. A similar oxygen uptake of 5 μl per 10 minutes was found in incubated control mixtures containing the above enumerated mixture without homogenate, and the above enumerated mixture containing tissue homogenate and cyanide in amounts giving a final concentration of $1x10^{-2}$M.

The rate of autoxidation of ascorbate in the presence of the convulsant and anticonvulsant agents was determined: (1) by extrapolation to zero tissue concentration from a series of 3 different tissue concentrations for each agent in concentrations of $1x10^{-3}$ to $1x10^{-5}$M; (2) by measuring the oxygen uptake of mixtures containing the agents, the substrate-mixture in the absence of tissue homogenate; and (3) by measuring the oxygen uptake of mixtures containing the agents, the substrate-enzyme mixture, and cyanide in amounts to give a final concentration of $1x10^{-2}$M. The oxygen uptake in 10 minutes averaged in most instances 5μl.

Calculation. The oxygen uptake due to autoxidation of the substrate was subtracted from the oxygen uptake of the substrate-enzyme (1 ml) mixture. The 3 point extrapolation to zero tissue concentration yielded straight lines and the intercept for the autoxidation rates was in almost all instances approximately 5 μl. The oxygen uptake of controls without convulsant and anticonvulsant agents was taken as 100%, and the oxygen uptake of mixtures containing the convulsant and anticonvulsant agents was expressed as a percentage of it. The effect of the agents on the activity of cytochrome oxidase was considered significant when the oxygen uptake deviated from 100% by more than twice the square root of the sum of the standard error of the mean of controls (mixtures without convulsant and anticonvulsant agents) and the standard error of the mean of a group of experiments (mixtures containing one concentration of one agent) $[2\sqrt{\text{S.E.}^2(\text{control}) \pm \text{S.E.}^2(\text{exp.})}]$.

Results. The results are summarized in Table I. The activity of cytochrome oxidase increased in the presence of the convulsant agents used (acetylcholine, caffeine, camphor, dichlorodiphenyltrichloroethane, methylsalicylate, pentamethylenetetrazol, picrotoxin, scilliroside, and strychnine) in concentrations of $1x10^{-3}$M and less. The greatest increase observed was over 150%. The activity of cytochrome oxidase was not modified significantly by the anticonvulsant agents used (hydantoin, methyl-phenyl-ethyl hydantoin, phenylhydantoinate sodium, phenobarbital, and tridione) in concentrations of $1x10^{-3}$M and less. The oxygen uptake in the presence of enzyme from various sources was different, but the effect of convulsant and anticonvulsant agents on the activity of cytochrome oxidase was approximately the same in the complete series using liver homogenate and in the few representative experiments using brain homogenate as the source of the enzyme.

Discussion. The observation that anticonvulsant agents fail to modify significantly the activity of cytochrome oxidase is in agreement with the observation that nembutal and similar agents depress neurones by some mechanism that is independent of azide and cyanide sensitive oxidative enzyme systems, *e.g.* the cytochromes(4).

The increased activity of cytochrome oxidase in the presence of the convulsant agents suggests that at some phase of their action the convulsant agents actively increase the cerebral oxidative processes. Theoretically it is possible that the well known increase of oxygen consumption of the brain during convulsive seizures is implicated as follows: (1) as a factor initiating seizures; and (2) as a part of the mechanisms through which each unit recovers after every discharge. Since

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TABLE I.
Effects of Agents on Activity of Cytochrome Oxidase (Rat Liver).

Agents	Oxygen uptake in mixtures containing the agents expressed in % of the oxygen uptake of the control* Concentration of the agents in mols: 1×10^{-3}	1×10^{-4}	1×10^{-5}
Acetylcholine	122†	140	160
Caffeine	163	152	127
Camphor	192	133	107
Dichlorodiphenyltrichloroethane	216	131	120
Methylsalicylate	183	131	124
Pentamethylene tetrazol	181	160	142
Picrotoxin	176	171	129
Scilliroside	238	202	189
Strychnine	139	173	139
Hydantoin	124	103	100
Methyl-phenyl-ethyl hydantoin	108	108	104
Phenobarbital	111	102	98
Phenylhydantoinate sodium	—	110	101
Tridione	120	102	97
Choline	193	96	98

* The standard error of the mean for each value was less than ±5%. Each value represents the average of 10 separate experiments.

† The ascorbate Qo_2 (dry liver after correction for incomplete homogenization) averaged 305. This value represents the 100%.

changes in the electrical activity of brain characteristic for convulsive seizures precede the increase of cerebral oxygen consumption (5), it is likely that the increase of oxygen consumption is not a precipitating factor of convulsive seizures.

The above presented results indicate, therefore, that convulsant agents activate at least 2 processes: (1) a non-oxidative mechanism that initiates the seizures; and (2) the oxidative recovery processes.

5. Davies, P. W., and Remone, A., *Research Publications of the Assn. for Research in Nervous and Mental Disease*, 1947, v26, 205.

Summary. 1. The activity of cytochrome oxidase increased in the presence of convulsant agents used in concentrations of $1x10^{-3}$M and less. 2. The activity of cytochrome oxidase was not significantly modified by the anticonvulsant agents used in concentrations of $1x10^{-3}$M and less. 3. Convulsant agents, therefore, activate at least 2 processes, namely, a non-oxidative mechanism that initiates the seizures and the oxidative recovery processes occurring in each unit during the convulsive seizures.

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Effect of Heparin and of Vitamin K on the Life Span of *Daphnia magna*.* (18035)

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The experiments here presented are a contribution to the pool of evidence tending to show that viscosity is related to fundamental life properties—such as parthenogenesis(1), mitosis(2), aging of egg cells(3), stimulation(4), etc. While no direct proof is presented that viscosity changes actually occurred in the presence of vitamin K and of heparin—which are the substances found in the present series of experiments to influence life span of Daphnia—the known relation of these substances to protoplasmic coagulation suggests the possibility that viscosity changes may be involved.

It has been previously reported that variations in the concentration of calcium and colligative ions are effective in altering the span of fertilizable life of egg cells like those of Arbacia(5) and Mactra(6). Inasmuch as the action of coagulants and anti-coagulants would appear to involve the calcium balance of protoplasm, it seemed likely that this type of substance should also influence the span of life.

For the experimental investigation of the above possibility the fresh water cladoceran, *Daphnia magna*, was utilized. Batches of 10 or, more usually, 20 specimens were placed in 50 cc of spring water to which various amounts of heparin (Hynson, Westcott and Dunning) or vitamin K (2-methyl-1,4-naphthoquinone, Eastman Kodak Company) had been added. Controls were retained in unaltered spring water. The solutions of vitamin K were made up with 1/50, 1/25, 1/10, 1/5, 1, 2, and 4 mg per 100 ml of water; percentages amounting to 1/50,000, 1/25,000, 1/10,000, 1/5000, 1/1000, 1/500 and 1/250 of 1%, respectively. Heparin was found effective in concentrations of 10, 20, 40, and 100 mg per 100 ml; or, 1/100, 1/50, 1/25, and 1/10 of 1%, respectively. Glass electrode pH measurements showed no significant differences in any of these solutions as compared with spring water. From March 13 to June 13, 1950 seven series of experiments were performed under a temperature range of 21 to 30°C. In any single experiment the temperature did not usually vary more than 3 or 4 degrees. Including the initial exploratory experiments, about 2000 specimens were employed, but the data given below is based on about half this number. The results were clear and consistent in all cases, and can be presented simply in tabular form. Since Daphnia is normally an actively swimming form, mobility is taken as a criterion of condition. It was found in the experiments that immobility is followed shortly by death and disintegration.

In Table I are given the results of a typical experiment. Twenty specimens were placed in each solution; a total of 180 for the experiment. In some cases specimens were transferred to water or heparin solutions after an initial exposure to vitamin K, and in others both substances were used simultaneously, as shown in the tables. Table II is a summation of all of the experiments.

It will be noted from the data that heparin, an anticoagulant, has a prolonging effect on the life span of *Daphnia magna* under the experimental conditions employed; while vitamin K sharply reduces the life span in concentrations down to 1/25 mg/100 cc. At

* I am indebted to Professor A. S. Chaikelis for some of the chemicals used in these experiments, and to Dr. William Tavolga for some of the living material.

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TABLE I.
Survival of Daphnia in Heparin and in Vitamin K Solutions.
Percentage Immobilized.

Time, hr	K/5	K/5 for 2½ hr then into water	K/5 for 2½ hr then into 20H	K/5 and 20H	K/5 and 40H	K/50	Control	20H	40H
2½	0	0	0	0	0	0	0	0	0
8½	95	0	0	100	100	0	0	0	0
24	—	25	25	—	—	0	0	0	0
46	—	55	40	—	—	0	0	0	0
70	—	100	65	—	—	50	45	20	0

Note: Symbol such as K/5 indicates a concentration of 1/5 mg of vitamin K per 100 ml. 20H indicates 20 mg of heparin per 100 ml of solution, etc.

TABLE II.
Survival of Daphnia.
Percentage Immobilized.

Solution	No. of specimens	Time in hr ½	2½	8½	24-28	36-44	70-100
K/50	40	0	0	0	0	0	48
K/25	80	0	0	0	26	55	100
K/10	110	0	0	65	82	100	—
K/5	80	0	0	95	95	100	—
1K	60	0	100	—	—	—	—
2K	20	50	100	—	—	—	—
4K	20	100	—	—	—	—	—
Controls	150	0	0	0	7	60	73
10H	40	0	0	0	0	35	30*
20H	80	0	0	0	0	0	40
40H	20	0	0	0	0	0	0
100H	80	0	0	0	0	10	60
100H and K/10	40	0	0	27	67	100	—

* Drop in percentage due to discontinuation of some experiments before this time in hours.
Note: Meaning of symbols as in Table I.

1/50 mg/100 cc there appears to be some reversal in the effect, that is, vitamin K in 1/50,000 of 1% concentration was somewhat stimulating as compared with the controls. It is of interest to point out that no food was provided, and that food availability is said to have, within limits, an inverse relationship to longevity of Daphnia(7). Also, since heparin promotes motility, as observed during the course of the experiments, it may well be—particularly at higher temperatures where oxygen solubility in water decreases—that oxygen availability becomes limiting upon the effect of heparin. This may account for the falling off of the effect at 100H as compared with 40H, which is noted in Table II. In this connection it should be pointed out that deepness of color in Daphnia seems to be related to the oxygen content of the water, but that haemoglobin is not regarded as an oxygen carrier in this animal(8).

By comparing the first 3 columns of Table I, especially at 8½ hours, it will be seen that the deleterious effect of vitamin K may be reduced or delayed by transfer to spring water or heparin solutions after an initial exposure to vitamin K. The simultaneous use of heparin and vitamin K does not seem to influence the effect of the latter except perhaps to a slight extent with very high comparative concentrations of heparin. See the effect of K/10 as compared with 100H & K/10 in Table II.

Summary. The life span of the common fresh water cladoceran, *Daphnia magna*, is shortened in the presence of vitamin K in the medium, and lengthened with heparin. The possibility of relationship to protoplasmic coagulation phenomena is pointed out.

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The Shwartzman Phenomenon. I. Inhibitory Action of Nitrogen Mustard (HN_2)* (18036)

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The widespread use of nitrogen mustards in the treatment of neoplastic disease and the recent report on the therapeutic trial of one of these agents in glomerulonephritis(1), prompted an attempt to learn something of the mechanism of their inhibitory action on the Shwartzman phenomenon as reported by Becker(2). Shwartzman(3) considers the phenomenon of local tissue reactivity to result from a state of increased vulnerability induced at the site of the preparatory injection by the bacterial filtrate. Becker suggested that the nitrogen mustard, HN_2, as well as benzol and x-ray, acted upon vascular endothelium and rendered the endothelial cells anergic, thus preventing them from reacting to the active principle of the bacterial filtrates.

It appeared feasible to examine this suggested action of HN_2 on the endothelium by taking advantage of the rapid fixation of nitrogen mustard in the organism as shown by Karnofsky *et al.*(4). This group reported that occlusion of the circulation to the intestine for 2-5 minutes during and after the injection of nitrogen mustard protected the intestine against the usual mucosal injury. They showed, further, that occlusion of the abdominal aorta protected the femoral marrow against the action of this agent. By means of a special clamp described below, the circulation to an area of skin on the rabbit's abdomen was completely interrupted during and after the intravenous injection of HN_2 for a time considered adequate for its fixation elsewhere in the tissues. Since it had been shown(4) that occlusion of the circulation to the lower limbs during and after the injection of nitrogen mustard protected the femoral marrow and prevented the severe granulocytopenia, it appeared desirable to study the effect of this protection on the inhibitory action of HN_2.

Methods and materials. 1. Rabbits of both sexes and of various strains, weighing 1800 to 3000 g were used. All preparatory injections were made in the R.U.Q. No rabbit in these experiments received more than one injection of nitrogen mustard. 2. *Nitrogen mustard.* Methyl bis (β-chloroethyl) amine HCl† was injected intravenously 4 days before the intravenous injection of bacterial filtrate in all experiments. The agent was freshly prepared in the concentration of 1 mg/ml of saline or distilled water before each injection; the dose was uniformly 2 mg/kg. 3. *Bacterial filtrates.* The filtrates used were meningococcus filtrate 44B‡ and one prepared from the saline washings of a 24-hour growth of *E. coli* on plain agar, after the general method of Shwartzman. The intradermal (preparatory) doses were 0.25 cc of the 44B and 0.5 cc of the *E. coli.* The intravenous doses were 1 cc/kg of a 1:10 dilution of the 44B and 1-2 cc/kg of the undiluted *E. coli.* While neither of the filtrates was titrated here, the 44B(5) was known to be potent in the dilution used and the results on the controls indicate that the coli filtrate was potent as well.

4. *Circulatory occlusion.* To prevent ac-

* The opinions and assertions contained in this article are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department.

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2. Becker, R. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 247.

3. Shwartzman, G., *Phenomenon of Local Tissue Reactivity*, Hoeber, N.Y., 1937.

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† The nitrogen mustard used in the earlier experiments was kindly supplied by Merck & Co. The larger part of the agent was furnished through the courtesy of Dr. H. E. Himwich of the Army Chemical Center.

‡ Kindly furnished by Dr. G. Shwartzman.

5. Shwartzman, G., personal communication.

TABLE I.
Inhibitory Effect of Nitrogen Mustard on the Shwartzman Phenomenon.
(A) Inhibitory effect of the mustard without other treatment.
(B) Effect of clamping the preparatory site.
(C) Effect of aortic occlusion.

Exp.	No. rabbits	HN_2 mg/kg	Duration stasis, min.	Intrav. filtrate	Negative	Positive*	Mild positive†	Dead before 4 hr
(A)	6	2	—	44B	5			1
	6	—	—	"		4	2	
(B)	6	2	10-14‡	*E. coli*	5			1
	4	—	—	"		4		
(C)	6	2	5-6‡	"		4	1	1

* Positive: lesion > 1 cm in diameter at 4-5 hr after intrav. inj.
† Mild positive: lesion definite, but < 1 cm in diameter at 4-5 hrs.
‡ Time indicated is measured from the completion of the HN_2 inj.

cess of HN_2 to a selected zone of skin on the abdomen, a special clamp§ was devised. The clamp consists of a patented locking wrench,‖ to the jaws of which a pair of 3/16 inch round steel rods, bent to a half circle of one-inch radius, was welded. The rods were shod with rubber tubing and applied to the tented relaxed skin and cutaneous maximus muscle of the ether-anesthetized rabbit. After the clamp had been locked into place, one cc of 5% fluorescein was injected intravenously and the abdomen examined under ultraviolet light. The complete absence of fluorescence in the occluded area was considered adequate evidence of circulatory occlusion. HN_2 was then injected and the clamp retained in place 10-14 minutes.

Occlusion of the aorta was done by a method based on a suggestion by Karnofsky, *et al.*(4). The aorta was obstructed by pressing a length of thick-walled rubber tubing on the anterior abdominal wall of the ether-anesthetized rabbit and seating it in the groove formed by the transverse processes of the lumbar vertebrae. The tubing was held in place, on the aorta, by wrapping a clinical blood pressure cuff, folded longitudinally, about the lower abdomen of the rabbit and thereafter inflating the cuff to about 260 mm Hg. After inflation, the HN_2 was injected and the occlusion maintained for varying periods.

§ Designed and constructed by Mr. J. F. Bronson, Master Mechanic, NMRI.

‖ Vise grip manufactured by Petersen Co., Dewitt, Neb.

Results. Results of following experiments are summarized in the table.

1. *Inhibitory effect of HN_2 without other treatment.* In a group of 11 rabbits, 5 of which received HN_2 4 days before the intravenous injection of filtrate, all controls were positive at 4-5 hours and all HN_2 animals were negative.

2. *Effect of excluding HN_2 from the site of the preparatory injection.* Clamping the local area with exclusion of HN_2 from the endothelium of the preparatory site did not prevent the inhibition in any of the 5 rabbits tested with potent filtrates.

3. *Effect of the occlusion of circulation to the lower limbs.* Of the 6 animals injected intravenously with potent filtrates 4 days after the aortic occlusion, one died 3 hours after the intravenous injection, and at this time showed petechial hemorrhages at the site of the preparatory injection. The remaining 5 animals showed mild to moderately strong positive reactions at 4-5 hours.

Discussion. The experiments reported here confirm the inhibitory action of HN_2 on Shwartzman phenomenon, but fail to support the hypothesis that the inhibition is mediated by action on the vascular endothelium, rendering it unable to respond to bacterial filtrates. Clamping the skin in such a manner as to deny HN_2 access to the area which was to be the site of the preparatory injection, failed to prevent the inhibitory effect. It has been further shown that although the HN_2 was permitted to reach the site of the preparatory injection in even greater concen-

tration than normal, the inhibitory effect was markedly reduced when the lower limbs were excluded from the action of the agent. On the basis of these findings, it is postulated that the inhibitory effect of HN_2 on the Shwartzman phenomenon is the result of the systemic rather than a local action of the agent.

Studies in progress on the peripheral blood and on the histologic changes in the bone marrow after the administration of HN_2, suggest that protection of the marrow may be a factor in the decreased inhibition seen after the aortic occlusion.

Summary and conclusions. 1. Inhibition of the Shwartzman phenomenon by HN_2 is confirmed. 2. Protection of the preparatory site from HN_2 fails to influence the inhibition. 3. Protection of the lower limbs from the action of HN_2 by aortic occlusion decreases or prevents the inhibition.

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Effects of X-Rays on Size of Yeast Cells.* (18037)

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Enlargement of cells after exposure to ionizing radiation has been observed in many types of biological material, although it is only infrequently mentioned in the literature (1-4). There are two general explanations for this enlargement. First, it may be due to swelling, which can be defined as enlargement resulting chiefly from selective increase of one of the cell constituents, namely water. Second, it may be due to nonselective and continued formation of protoplasm and other cell constituents with a simultaneous slowing of the division rate.

The purpose of this work has been to determine whether the increased size of x-irradiated yeast cells is selective or nonselective. To obtain evidence bearing on this question, irradiated and nonirradiated cells have been compared with respect to two properties. The first is their specific gravity. This quantity is a fair indication of the amount of nonaqueous materials in the cells, since most of these are heavier than water. If the irradiated cells have a significantly lower specific gravity than the nonirradiated ones, this indicates that enlargement is at least partly due to simple swelling. On the other hand, if there is no significant difference between the specific gravities, this indicates that enlargement is due to nonselective increase in amounts of aqueous and nonaqueous material. The second basis of comparison is total nitrogen content, which may be taken as a rough index of the amount of protoplasm in the cells. If the nitrogen content of the irradiated cells is significantly lower than that of the controls, this indicates selective enlargement whereas substantially identical nitrogen contents are evidence for nonselective enlargement.

The yeast was a single-cell strain of *Saccharomyces cerevisiae* (Schenley No. 52). The cells were grown at 28°C in an aqueous solution containing 2% glucose and 0.3%

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4. Failla, G., *Am. J. Roentgenol.*, 1940, v44, 649.

yeast extract. The source of radiation was a General Electric Maximar X-ray machine operated at 230 kv and 15 ma. A yeast culture to be irradiated was placed in a 15 ml Carrel flask and the flask was placed close to the window of the x-ray tube. No filter was used except the tube wall and window and the wall of the flask. The dose rate was approximately 850 r/min as measured with a Victoreen condenser r-meter. No attempt was made to maintain constant temperature or to agitate the material during irradiation.

As a preliminary to the studies on specific gravity and nitrogen content, irradiated and nonirradiated cells were observed under the microscope. When cells were grown in liquid medium, irradiated in the nondividing condition, and transferred to fresh liquid medium, a very characteristic pattern of changes occurred. During the first few hours after irradiation, the cells appeared normal or nearly so, except for an occasional cell that was small and shrunken. A large fraction of the cells formed doublets with a peculiar dumbbell shape. This has been observed by Lacassagne(1) and many others. During the next few hours there was a gradual increase in cell size, most of the cells being paired or in clusters of four to eight. The cells that did not enlarge were nearly all single. The maximum cell size was reached 6 to 8 hours after the onset of growth; this represents a period of 3 or 4 normal division times. During the next 4 hours the swollen cells gradually disappeared and large numbers of normal cells appeared. After 20 hours the large cells were very rare. All of these changes could be stopped by low temperature or by exhaustion of food in the medium.

In order to follow the history of single irradiated cells, cultures were grown in liquid medium diluted to 1% of the usual concentration of glucose and yeast extract, and the cells were transferred directly from the Carrel flask to the free surface of a level plate of potato-dextrose agar. A block of agar about 1 cm square was then transferred to a special ruled slide with the cells and the rulings on substantially the same plane. To prevent evaporation the square of agar was covered with a depression slide taped to the ruled slide. These agar cultures were incubated at 28°C, and several hundred individual cells and their descendants were followed until overgrowth occurred. Practically none of the cells disintegrated. Most of them progressed to the doublet stage and then started to enlarge without dividing. After a delay of several hours most of the cells were able to resume a normal division rate and returned to normal size. The large colonies derived from rapidly recovering cells soon outgrew the remainder of the colonies. The longer the delay before normal division returned, the more enlarged the cells became. A few cells were able to undergo only 3 or 4 divisions, and small colonies of giant cells resulted. Some of these colonies of giant cells gradually disappeared, presumably by lysis, while others persisted for long periods without change. The single cells which failed to enlarge were able to divide after a delay (10 hours or more).

In order to compare the specific gravities, the nitrogen contents and the mean volumes of irradiated and nonirradiated cells and their descendants, the yeast samples were cultured in liquid medium at 28°C and the following quantities were determined: 1. The specific gravity of the total suspension (ρ_s) and of the medium alone (ρ_m). These measurements were made with a kerosene-bromobenzine gradient tube, as described by Linderstrøm-Lang and Lanz(7) and modified for use with yeast by Atkins *et al.*(8). The standard solutions consisted of copper sulfate in distilled water and were kept under oil. 2. The fraction (F) of the culture volume occupied by the cells. This was determined by drawing a well-mixed sample of the culture into a calibrated Van Allen blood volume index tube and centrifuging until the packed cell volume became constant. 3. The amount of nitrogen per mm^3 of cell suspension (Q_s) and of medium alone (Q_m). These quantities were determined by the micro-Kjeldahl technic devel-

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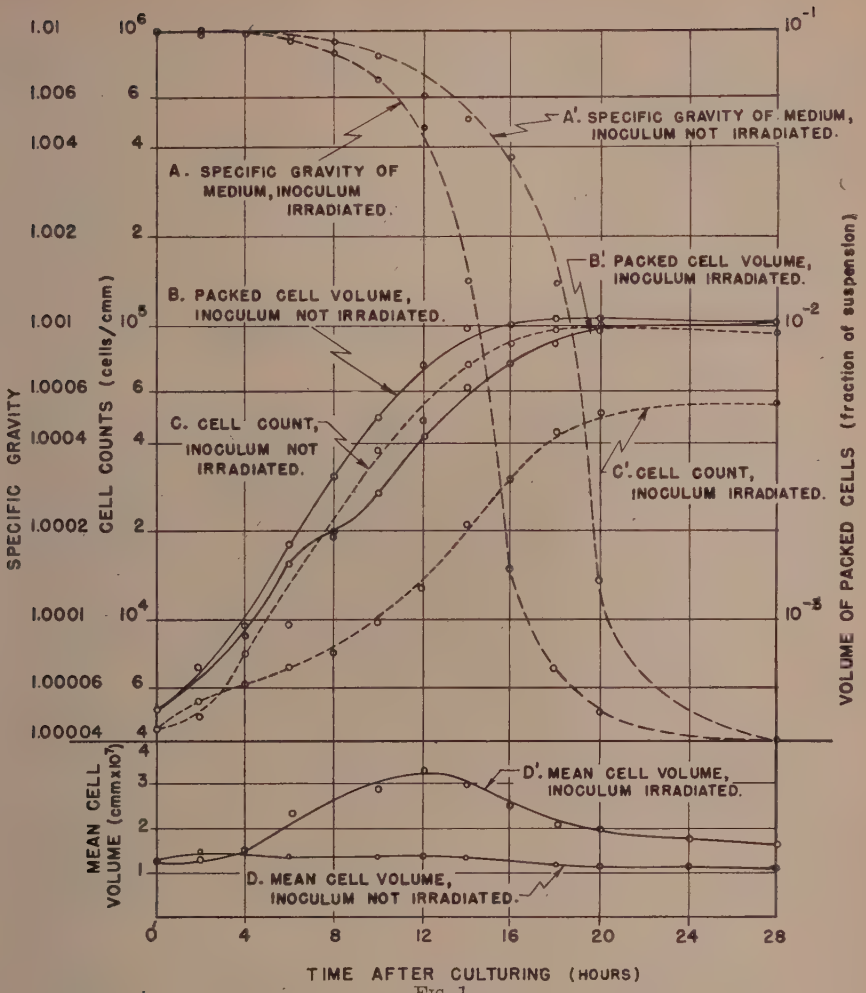

FIG. 1.

Time courses of changes in properties of yeast suspensions grown from x-rayed and non-irradiated cells.

oped by Linderstrom-Lang and coworkers and modified by Doyle and Omoto(6). 4. The number N of cells per mm³ of suspension. This figure was obtained by hemocytometer count of an undiluted sample of cell suspension.

From these data the following quantities were calculated: 5. The specific gravity of the cells (ρ_c) was derived from the relationship

$$F\rho_c = \rho_s - (1 - F)\rho_m \qquad (1)$$

6. The nitrogen content of the cells in micrograms per cmm, was

$$Q_c = \frac{Q_s - Q_m}{F} \qquad (2)$$

7. The mean cell volume in mm³ was equal to F/N.

In preliminary experiments, in which graded x-ray doses were used, it was found that maximal enlargement of cells could be produced by 20,000 r. Accordingly this dose was used in all subsequent experiments.

Further preliminary experiments were performed to determine the time course of cer-

tain changes which occur when normal and irradiated cells grow and multiply in liquid medium. A stock culture of cells was allowed to grow until the glucose in the liquid medium was exhausted. A sample was irradiated in a sterile Carrel flask, and a control sample was handled identically except for irradiation. From each of these 2 samples, 1 ml of cell suspension was immediately transferred to 25 ml of fresh sterile liquid medium in a 125 ml Erlenmeyer flask and incubated at 28°C. For 20 hours thereafter, samples were taken at intervals from both of these cultures for determination of cell count, packed cell volume and specific gravity of the medium. The results are plotted in Fig. 1. The time courses of decrease in specific gravity of the cell media are shown by the dashed curves A and A′. The chief conclusion to be drawn from these curves is that the irradiated cells and their progeny (curve A′) lag about 4 hours behind the controls in their removal of glucose from the medium.‡ The packed cell volumes (solid curves B and B′) increase at substantially the same rate, except that, during a 4-hour period starting about six hours after culturing, the irradiated cells and their descendants increase much more slowly than the controls (note kink in curve B′). This temporary lag may be due to lysis of some of the swollen cells. The cell counts (curves C and C′) show that during the first two hours the irradiated cells divide slightly more rapidly than the controls (this was consistently observed) but that thereafter they divide more slowly. From the packed cell volumes and the cell counts, the mean cell volumes can be calculated. It will be noted that the cells derived from nonirradiated cells (curve D′) remain substantially constant in mean volume but that the irradiated cells and their progeny (curve D) are maximal in mean volume about 12 hours after culturing and, with the exception of the first 4 hours, are always larger than the controls.

In each of the final experiments several different measurements had to be made on the culture derived from irradiated cells and on its control. Since the cultures changed so rapidly during the logarithmic growth phase, good comparative measurements would have been very difficult to obtain during this period. This difficulty was overcome by so adjusting the amount of irradiated (or control) suspension transferred to fresh medium that the glucose became exhausted and growth stopped when the mean cell volume was approximately maximal. In this way samples of large cells could regularly be obtained at the time when the difference between the specific gravity of the suspension and that of the medium was maximal. The cell counts and the packed cell volumes were first determined. If the mean cell volume of the irradiated cells and their progeny was at least twice that of the controls, each culture was divided into 2 portions, one of which was centrifuged to obtain a sample of cell-free medium. The specific gravity and the nitrogen content of each sample were then determined, each measurement being performed in triplicate.

Two complete experiments were carried out as just described, and the experimental measurements are presented in Table I along with the quantities which can be calculated from them—namely the specific gravity of the cells alone and the nitrogen content of the cells. It will be noticed that in both experiments the specific gravity and the nitrogen content of the irradiated cells and their progeny are slightly but consistently less than the controls. The individual differences are probably within the experimental error of the measurements of specific gravity differences and of nitrogen content, the triplicate determinations of which deviate as much as 5 per cent from the mean. The consistency of direction of the differences is probably due to the tendency of the enlarged irradiated cells to clump and thus fail to pack as well as the controls. The corresponding values of packed cell fraction F would thus tend to be high, and the calculated values of nitrogen content (Q_c) and specific gravity (ρ_c) would therefore tend to be low, as observed. However, these discrepancies, compared to the large differences in cell volume, are small, and

‡ The decrease in specific gravity is a fairly good index of glucose depletion despite the complication of alcohol formation.

TABLE I.
Comparison of Cells Derived from Normal and Irradiated Yeast Cells.

	Exp. 1		Exp. 2	
	Normal	Irradiated	Normal	Irradiated
Cells per cmm of culture (N)	101,300	46,400	153,000	69,000
Fraction of culture volume occupied by packed cells (F)	0.0108	0.0115	0.0147	0.0134
Mean cell volume in cmm $\times 10^7$ (F/N)	1.07	2.48	0.96	1.94
Nitrogen content of cell suspension in μg/cmm (Q_s)	0.318	0.323	0.382	0.384
Nitrogen content of medium in μg/cmm (Q_m)	0.094	0.109	0.091	0.135
Nitrogen content of packed cells in μg/cmm (Q_c)	20.7	18.6	19.8	18.6
Specific gravity of suspension (ρ_s)	1.000674	1.000632	1.000787	1.000648
" " " medium (ρ_m)	1.000010	1.000016	0.999886	0.999861
Specific gravity of cells (ρ_c)	1.061	1.054	1.061	1.060

one may regard the data for the irradiated and control cultures to be substantially equal.

Accordingly it is to be concluded that, in the case of the yeast cell, the enlargement observed after irradiation is chiefly if not entirely due to a nonselective increase in water and nonaqueous constituents. This nonselective increase probably proceeds through normal mechanisms which are much more resistant to irradiation than is the mechanism of cell division.

So far as comparisons can be made, a similar state of affairs was produced in the protozoan *Bodo caudatus* by Robertson(2). However, the enlargement of certain types of cells, *e.g.* erythrocytes(5), after irradiation seems to be due almost entirely to uptake of water (swelling). Failla(4) has discussed this phenomenon and has offered a theory to explain it.

Summary. When yeast cells were exposed to 20,000 r of x-rays and cultured in liquid medium, they and their descendants attained a mean cell volume more than twice that of cells derived from nonirradiated controls. On the other hand, there were no corresponding differences in specific gravity and nitrogen content per unit of cell volume. It is accordingly concluded that enlargement of yeast cells after irradiation is largely a nonselective increase in cell constituents rather than a swelling due mostly to uptake of water.

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Survival of *Trichomonas vaginalis* in Vaginal Discharge.* (18038)

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The transmission of *Trichomonas vaginalis* is partially dependent upon the ability of the flagellates to survive in natural discharges. Vazquez-Colet and Tubanqui(1) reported that *Trichomonas vaginalis* would survive in a semi-dry state for 6 hours and Swift(2) stated that the trichomonads could be cultured from vaginal discharge that had been dried for 3 hours. Kirby(3) reported that most trichomonads in vaginal discharge under paraffin-sealed cover-slip preparations kept at room temperature survived for 24 hours

* Aided by a grant to the Microbiology Research Fund, by Protecto Products Co.

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2. Swift, B. H., *Med. J. Australia*, 1937, v1, 123.

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and a few more were active after 5 days. Although these observations indicate that trichomonads may survive in drying or sealed vaginal discharges at room temperature for a considerable time, they do not establish the fact that they will multiply in serial cultures following such survival. It was considered that more exact information concerning the resistance of *Trichomonas vaginalis* under natural conditions of drying in the vaginal discharge and their reproduction in serial cultures following such drying would provide pertinent data concerning the possibility of their transmission by other than venereal methods.

Materials and methods. The discharge used in this study was collected from 50 untreated patients with Trichomonas vaginitis. A sample was taken from the interior surface of a sterile speculum immediately upon removal from the patient, and examined microscopically for the presence of trichomonads. (1) One-tenth cc was inoculated immediately into 8.0 cc of standard culture medium and served as a control. This was incubated at 37°C and examined after 48 hours, at which time transplants were made to fresh media for subcultures. (2) Five-tenths cc of the discharge was transferred for experimental study to an area within a limiting circle, 1.5 cc in diameter, drawn with a wax pencil on the enameled surface of a small wooden block. Each drop of discharge was examined at intervals of 10, 20, 30 and 45 minutes, and of 1, 2, 3, 4, 5, 6 and 7 hours respectively when a small portion of the material was removed and examined for active trichomonads. Simultaneously an equivalent amount was transferred to a culture tube of medium, incubated and observed after 48 hours for active flagellates. Transfers were subsequently made to other culture tubes and the fact established that the trichomonads were capable of growth in serial transfer. The drying droplet was sufficiently moist for the first 5 hours to permit the preparation of a routine smear, but the portion of the droplet remaining on the block, at both the 6 and 7 hour intervals, had dried to the point where it was first necessary to add saline to the sample in order to make a smear on a slide for microscopic examination. The culture medium used was a Casamino acid medium (Difco–Lash's Serum Medium) described by Lash(4,5). In this study 1.0 g of cysteine hydrochloride per 1000 cc was added, in an attempt to decrease through reduced oxygen tension the bacterial overgrowth, while establishing cultures(6-8). Penicillin and streptomycin were not used, since no attempt was being made to retain the trichomonads in bacterial free culture.

Results. Table I displays the results of studies on the discharges from 50 cases of vaginitis which were positive for *Trichomonas vaginalis* by the procedures outlined under *Materials and methods.*

It will be observed that the trichomonads remained active for a period of at least 45 minutes in all 50 of the drops of discharge studied. Also that growth occurred from all 50 of these samples when they were transferred to suitable culture media. After 6 hours, active trichomonads were found in material from only 2 of the 50 cases and only one of these survived in culture. The decrease in survival times of trichomonads between these two periods is displayed in Fig. 1.

Here it is noted that there is a gradual diminution in survival of trichomonads as the time increases. Little difference is observed between the number of samples in which active trichomonads occurred in direct smear and the number established in culture. One

TABLE I.
Results of Direct Smear Examination and Culture of Droplets from Vaginal Discharge.

Time	Direct smear motility	48-hr culture growth
10-45 min.	50	50
¾-1 hr	48	49
1-2 "	45	46
2-3 "	27	29
3-4 "	12	13
4-5 "	2	3
5-6 "	2	1
6-7 "	0	0

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7. Wadsworth, A. B., *J. Inf. Diseases*, 1943, v73, 95.
8. Ward, T. G., PROC. SOC. EXP. BIOL. AND MED., 1941, v48, 19.

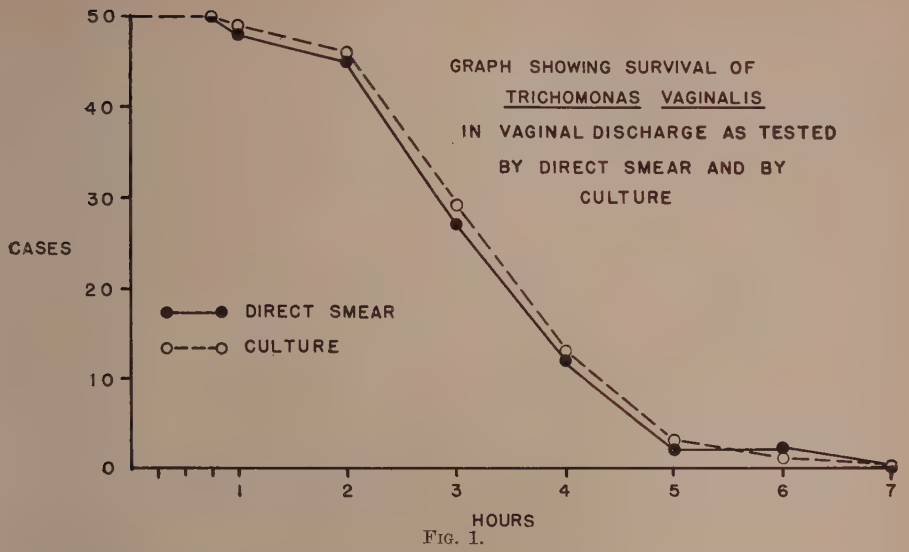

FIG. 1.

more sample at the one hour period yielded a positive culture than was demonstrated to show motility by direct mount. At the 6-hour period two cases still exhibited motility but only one of these was established in culture. No active trichomonads were observed and no positive cultures procured from any of the specimens examined at the 7-hour period.

It is thus concluded that the trichomonads which exhibited motility following the observed periods of standing within a drying drop of vaginal discharge, with one exception, were also capable of multiplying in culture.

Discussion. The distribution and transmission of *Trichomonas vaginalis* are discussed in detail in the excellent monograph by Trussell(6). He points out that one out of every 4 or 5 adult women harbors the parasite. He also accepts the conclusion that *Trichomonas vaginalis* is the only trichomonad associated with Trichomonas vaginitis. Since neither *T. hominis*(10,11) nor *T. elongata*(12) is capable of becoming established in the vagina, there would appear to be no further need for considering the intestinal and oral trichomonads as potential etiologic agents of vaginal trichomoniasis.

Since *Trichomonas vaginalis* is frequently recovered from the genito-urinary tract of both males and females, sexual intercourse is generally accepted as an important method of transmission. However, this method does not appear to explain all cases of infection in adults, cases reported from infants and children(13-15) nor multiple infections in the same family, especially of mothers and children(15-17). It is accordingly quite natural to consider that certain fomites *e.g.* towels, wash cloths, toilets and bath tubs also may function in the transmission of this parasite.

9. Berkman, S., PROC. SOC. EXP. BIOL. AND MED., 1940, v44, 68.

10. Kessel, J. F., and Gafford, J. A., *Am. J. Obst. and Gynec.*, 1940, v39, 1005.

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16. Stein, I., and Cope, E., *Am. J. Obst. and Gynec.*, 1933, v25, 819.

17. Katsunuma, S., *Bull. Soc. Path. Exot.*, 1924, v17, 216.

The current study was undertaken to confirm previous observations with reference to the length of time that Trichomonas vaginalis was capable of surviving in a droplet of vaginal discharge under natural conditions and also whether it was capable of multiplying in culture at the end of each test period. It is observed in Table I and Fig. 1 that trichomonads survived for 45 minutes in the discharges from all cases tested, that in 29 or 58% they survived for 3 hours and that survival was demonstrated in only 2 or 4% at the end of 6 hours.

Trussell(6) raises the question of the survival of trichomonads following desiccation. In the current study the drying droplets of discharge were sufficiently moist up to the 5-hour period to permit transferring by pipette to a microscope slide or culture tubes the amount of material required for testing. At the 6- and 7-hour intervals, however, the specimens had become so dry that it was necessary to add saline to the block before the material could be transferred. Since only one of the 50 specimens tested at 6 hours was positive by culture and none of the 7-hour specimens yielded either motile flagellates or positive cultures, it is concluded that trophozoites of *Trichomonas vaginalis* will not withstand complete desiccation.

The observations here presented, however, do indicate that trichomonads in droplets of vaginal discharge, deposited under natural conditions may survive sufficiently long to permit transfer to another individual where they may again be established. Although actual experimental transfer of the recovered trichomonads has not been made to volunteers during this study, previous experiments by one of the authors(10) and also by other investigators(6,18) have demonstrated that *Trichomonas vaginalis* established in culture can be implanted and produce vaginal trichomoniasis. In view of these findings careful personal hygiene is indicated.

Summary. The survival of *Trichomonas vaginalis* was determined by studying discharge from 50 untreated patients with vaginal trichomoniasis. Droplets of the discharge were placed on the enamel surface of wooden blocks and tested under natural conditions of drying at room temperature.

Tests at intervals ranging from 10 minutes to 7 hours were made by comparing the activity of the flagellates in direct microscopic examination with their ability to multiply in serial cultures. A close correlation was observed between the two procedures. The trichomonads survived in 100% of the tests at 45 minutes, in 96% at 1 hour, in 56% at 3 hours and in 4% at 6 hours. No survival was demonstrated at 7 hours.

These data, together with experimental and epidemiologic reports of other workers indicate that fomites contaminated with *Trichomonas vaginalis* may, under natural conditions, act as agents in the transmission of vaginal trichomoniasis.

18. Hesseltine, H. C., Wolters, S. L., and Campbell, A. J., *J. Inf. Dis.*, 1942, v71, 127.

Received June 19, 1950. P.S.E.B.M., 1950, v74.

Relation of Cortisone Pretreatment to Mobilization of Lipids to Liver by Pituitary Extracts.*† (18039)

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Since the initial report of Best and Campbell(1), it has been shown in many labora-

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† A preliminary report of this work was made before the Division of Biological Chemistry of the American Chemical Society, Philadelphia, April 11, 1950.

tories that crude pituitary extracts are able to cause a rapid increase in the amount of fat in the liver. It has further been demonstrated(2,3) that this accumulating fat originates from the fatty depots of the body, the overall phenomenon therefore consisting of a mobilization of depot fat to the liver. A number of attempts have been made to attribute this property of pituitary extract to various of the known pituitary hormones, but to date no definite evidence has been presented to prove that any one or another of the known pituitary hormones is responsible for the mobilization of fat to the liver.

Data presented elsewhere(4-7) have indicated that the property of mobilization of fat to the liver cannot be ascribed solely to the action of adrenocorticotrophic hormone (ACTH) acting via the usually accepted route, *e.g.*, by stimulation of the secretory activity of the adrenal cortex. For example, it has been shown(5-7) that none of the available adrenocortical steroids (including cortisone) is able to cause accumulation of fat in the liver. On the other hand, there is almost complete‡ agreement(5,6,7,10,11,12) that total adrenalectomy completely prevents fat mobilization by pituitary preparations which are active, in this respect, in intact animals. Thus there exists a considerable contradictory dilemma as to the function of the adrenal cortex in relation to mobilization of fat.

The data here presented provide a significant clue as to the mode of participation of the adrenal cortex. These data show that in order to produce mobilization of depot fat to the liver two hormonal factors are required: (a) an adequate supply of circulating 11-oxygenated steroid (whether endogenous or exogenous) before and/or during the time of fat mobilization; and (b) the actual triggering substance, an appropriate pituitary extract or hormone. The latter is apparently neither ACTH nor growth hormone, or alternatively and less likely, each of these hormones has the ability to cause the mobilization of fat in appropriately prepared animals. These data further conclusively prove that whatever the identity of the pituitary substance involved, its action in triggering mobilization of fat to the liver is not mediated via the adrenal cortex.

Experimental. Adult female mice of the Carworth Farms CFW strain were used as experimental animals and were killed by cervical fracture at the end of a 7 hour fasting period. Pituitary preparations, when administered, were given as a single subcutaneous injection at the start of the fasting period. When treatment with cortisone was used, it was begun 3½ days prior to sacrifice of the animal. The steroid was administered in 2 daily subcutaneous injections of 0.5 mg each (usually as a suspension of the steroid

1. Best, C. H., and Campbell, J., *J. Physiol.*, 1936, v86, 190.

2. Barrett, H. M., Best, C. H., and Ridout, J. H., *J. Physiol.*, 1938, v93, 367.

3. Stetten, D., and Salcedo, J., *J. Biol. Chem.*, 1944, v156, 27.

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6. Levin, L., Abst. of Proc., First Int. Cong. Biochem., Cambridge, England, 1949, p. 393.

7. Levin, L., Abst. of Papers, 117th Meeting, Am. Chem. Soc., Philadelphia, 1950, p. 13c.

‡ The only exceptions known to us are the papers of Szego and White(8) and of White(9), both dealing with the same data, in which it is reported that mobilization of fat to the liver was obtained after administration of incompletely purified growth hormone to adrenalectomized as well as to intact mice. We have not been able to confirm either finding with the 3 growth hormone preparations we have used to date. We believe that we can explain the discrepancy between results obtained in intact mice in the 2 laboratories. This will be done in a later publication. Preliminary results, now being verified, also appear to offer a possible explanation of the discrepancy between the findings in adrenalectomized mice by Szego and White on the one hand and those obtained in this and a number of other laboratories on the other hand.

8. Szego, C. M., and White, A., *Endocrinology*, 1949, v44, 150.

9. White, A., *Recent Progress in Hormone Research*, vIV, p. 153, Academic Press, Inc., New York, 1949.

10. Fry, E. G., *Endocrinology*, 1937, v21, 283.

11. McKay, E. M., and Barnes, R. H., *Am. J. Physiol.*, 1937, v118, 525.

12. Payne, R. W., *Endocrinology*, 1949, v45, 305.

TABLE I.

Effect of Cortisone Pre-treatment on Liver Fat Response of Mice to Various Pituitary Preparations.

Exp. No.	Treatment‡	No.	Mean body wt, g	Liver wt		Liver fat	
				Mean ± σ* g/100 g BW	Change, %	Mean ± σ* mg/100 g BW	Change, %
1	Intact controls	41	20.8	5.53 ± .16		349 ± 8	
		Adrenalectomized.					
2	Untreated controls	7	21.8	5.00 ± .51	— 9	257 ± 18	—26
3	Cort. only	10	21.0	5.56 ± .25	+ 1	389 ± 14	+11
4	20 mg P18	5	24.9	4.33 ± .25	—22	281 ± 22	—19
5	Cort. + 20 mg P18	8	21.7	6.01 ± .24	+ 9	651 ± 43	+87
6	5 mg ACTH 71-2(50)c	6	23.3	5.36 ± .34	— 3	230 ± 17	—34
7	Cort. + 5 mg ACTH 71-2(50)c	8	24.8	5.52 ± .38	0	514 ± 24	+47
8†	5 mg ACTH 32-D	12	22.2	4.53 ± .16	— 6†	265 ± 13	—21†
9	Cort. + 5 mg ACTH 32-D	4	23.4	5.37 ± .21	— 3	623 ± 31	+79
10	Cort. + 5 mg GH R-95	8	23.7	5.87 ± .25	+ 6	638 ± 28	+83
11	Cort. + 5 mg GH 12PKR3	9	22.6	6.02 ± .14	+ 9	603 ± 38	+73
		Intact.					
12	Cort. only	10	24.5	6.12 ± .25	+11	386 ± 22	+10
13	5 mg GH #R-95	9	22.6	5.46 ± .28	— 1	354 ± 23	+ 1
14	Cort. + 5 mg GH #R-95	4	24.1	5.35 ± .31	— 3	552 ± 37	+58
15	5 mg GH #12PKR3	9	24.7	5.26 ± .22	— 5	338 ± 20	— 3
16	Cort. + 5 mg GH #12PKR3	4	23.9	5.35 ± .05	— 3	629 ± 26	+80

* The value for σ was calculated as $\sigma = \sqrt{\Sigma d^2/N(N-1)}$.

† The mice of this group were of another strain and therefore these results are compared to those of 36 control mice of that strain.

‡ All animals sacrificed at end of 7-hr fasting period. "Cort." indicates pre treatment with 3.5 mg cortisone (suspension) in 7 equal subcutaneous doses during the 3½ days immediately preceding sacrifice. In adrenalectomized animals this treatment coincides with the period following adrenalectomy. Pituitary preparations were administered as a single subcutaneous injection at the start of the fasting period. The pituitary preparations used were the following:

P18—A crude, water soluble fraction from sheep pituitary glands.

ACTH 71-2(50)c—An Armour Laboratories preparation described as having 20% of the potency of the Armour ACTH Standard LA1A when assayed by the adrenal ascorbic acid depletion method. Substantially free of growth hormone.

ACTH 32-D—Armour preparation having 78% of the potency of LA1A. Negligible growth hormone content. 0.05 USP oxytoxic units and 0.1 pressor units per mg.

GH R-95—Armour preparation assaying 25 Evans growth units per mg.

GH 12PKR3—Approximately equivalent to the electrophoretically and ultracentrifugally homogeneous Armour standard growth hormone 22KR2. Essentially free of ACTH activity.

in 20% alcohol), the total dose thus being 3.5 mg during the 3½ days immediately preceding autopsy. In adrenalectomized animals this period coincided with the period of adrenalectomy, the treatment being initiated immediately post-operatively. Such animals also were given 1% sodium chloride to drink instead of water. Livers were removed immediately after sacrifice and total lipid content was determined by a modification of the method of Channon, Platt and Smith(13). Figures for liver lipid are presented as milligrams of liver "fat" per 100 g of body weight. However, it is emphasized that the results are essentially the same if liver lipid data are compared in terms of total quantity of fat per liver or as percent of fat in the wet liver.

Pertinent data are presented in Table I. It may be seen (Exp. 2) that a 3½ day post-adrenalectomy period results in a loss of liver fat as compared to the values obtained from intact controls. The loss (26%) suffered by the presently reported small group of adrenalectomized controls compares favorably with that noted in 2 larger groups of adrenalectomized mice of another strain. The liver fat content of the latter 2 groups (19 females and 17 males, respectively) was lower by 15 and 19%, respectively, than those of appropriate intact controls of the same strain.

The data further demonstrate (Exp. 3) that treatment with cortisone (1 mg per day for 3½ days) during the post-adrenalectomy interval prevents the loss of liver fat which otherwise follows adrenalectomy and even causes a very small increase. This increase is identical to that seen (Exp. 12) in intact mice similarly treated with cortisone. It may be added that we have obtained somewhat similar results by treatment with comparable doses of desoxycorticosterone acetate or of 11-dehydrocorticosterone. We have interpreted the small increases to be the result of non-specific supportive therapy rather than as a direct effect of the adrenocortical steroid on mobilization of fat.

Our uniform experience to date has been that pituitary extracts, regardless of variety, are unable to elicit an increase in liver fat on otherwise untreated adrenalectomized mice. One example of many such negative responses may be seen in the results obtained by treatment of adrenalectomized mice with the impure pituitary extract P18 (Exp. 4). It may be parenthetically added that the same preparation is quite active in *intact* mice, the same dose causing the liver fat content to increase by 44%.

The effect of cortisone treatment on the response of adrenalectomized mice to pituitary extract is shown in Exp. 5, Table I. In the animals pre-treated with cortisone the same pituitary extract discussed immediately above, (P18), produced an excellent response, the liver fat level being increased by 87% as compared to that of intact fasted control mice. These results are interpreted to indicate that (a) the fat-mobilizing action of the pituitary extract is not mediated by the adrenal cortex but (b) the extract is not able to cause fat mobilization unless the test animal has been supplied (from exogenous or endogenous sources) with adequate amounts of adrenocortical hormone.

Similar effects of pre-treatment with cortisone were obtained in adrenalectomized mice treated with ACTH. It is to be noted that neither of the two ACTH§ preparations (No. 32-D and 71-2(50)c) used in the presently reported experiments caused any increase in the liver fat content of otherwise untreated adrenalectomized mice (Exp. 6 and 8). It is of interest that these two ACTH preparations differed considerably in their potency as assayed by the adrenal ascorbic acid depletion method and, in intact mice, showed a proportional difference in ability to cause mobilization of fat to the liver. Thus, when assayed by depletion of ascorbic acid,§ preparation No. 32-D was approximately 4 times

13. Channon, H. J., Platt, A. P., and Smith, J. A. B., *Biochem. J.*, 1937, v31, 1736.

§ The ACTH and growth hormone preparations used in these experiments were generously furnished by the Armour Laboratories through the cooperation of Drs. John R. Mote and Irby Bunding. The potencies with regard to ACTH content (by adrenal ascorbic acid depletion) and to growth-promoting activity are those reported to the writers by the Armour Laboratories.

as active as No. 71-2(50)c. In intact mice, a 5 mg dose of ACTH 32-D caused a 78% increase in liver fat while a similar dose of No. 71-2(50)c yielded but a minimal response (8% increase). In adrenalectomized mice pre-treated with cortisone (Exp. 9 and 7), liver fat increases of 79 and 47%, respectively, were obtained with these two ACTH preparations. This result with preparation No. 71-2(50)c is particularly interesting in view of its inactivity in intact mice and is emphasized here because it demonstrates the sensitizing effect of cortisone pre-treatment particularly when used with pituitary preparations low in or completely lacking adrenocorticotrophic activity. These results not only confirm the conclusions drawn from the experiments with crude pituitary extract *(vide supra)* but also suggest that it is not the adrenocorticotrophic hormone, *per se,* which triggers the fat mobilization reaction.

Experiments with growth hormone preparations lead to similar conclusions. None of the 3 such preparations tested in this laboratory to date, when administered in doses up to 5 mg per mouse, has produced any increase in liver fat content *even in intact mice*‖ (Exp. 13 and 15). Likewise, the 2 of these 3 growth hormone preparations which have been administered to otherwise untreated adrenalectomized mice have proven completely inactive in mobilizing fat to the liver. However, when administered to intact (Exp. 14 and 16) or adrenalectomized (Exp. 10 and 11) mice which had been pre-treated with cortisone, the two growth hormone preparations tested in this fashion both produced marked increases in liver fat content. It should be emphasized that these preparations are reported§ by the manufacturer to be inhomogeneous but of a potency, measured by growth response, equal to that of pure growth hormone. It is also of considerable significance that, when assayed by the adrenal ascorbic acid depletion method, these preparations were found to contain very little, if any, demonstrable ACTH.

The results reported herewith, as well as those published by Payne(12), thus assume considerable significance with respect to the endocrine mechanism of fat mobilization. It has been shown in this and other laboratories that adrenalectomy completely abolishes the fat mobilizing response to administered pituitary preparations. This, plus the fact that many ACTH preparations are active in intact animals in this respect, has led to the impression that fat mobilization is a manifestation of ACTH activity mediated by the adrenal cortex. However, previous results(4-7) as well as the presently reported experiments throw considerable doubt on such an hypothesis. It now seems quite evident that a variety of pituitary preparations (crude extracts, ACTH or growth hormone) are able to cause mobilization of depot fat to the liver without the mediation or even the presence of the adrenal cortex provided the animal is adequately pretreated with cortisone (or perhaps other adrenal steroids).

Furthermore, the results here reported make it appear quite unlikely that fat-mobilization is a specific property of ACTH or of growth hormone. The fact that both ACTH and growth hormone, each substantially devoid of the other, are active in this respect (provided sufficient circulating cortisone has been available to the animal) and the fact(14) that the fat mobilizing potency of ACTH is not always proportional to the activity as measured by ascorbic acid depletion, both indicate that ACTH is not the causative factor.

‖ The explanation for the disagreement between these results and those reported from several other laboratories is not readily apparent. It may be pointed out, however, that the preparation used by Szego and White(8,9) was reported to be impure and an explanation may be forthcoming as indicated in footnote ‡. Also, it is perhaps significant to note that Payne(12) and Weil and Ross(15) used the same preparation (No. 3PKR3 of Armour Laboratories). Li *et al.*(16), on the other hand, used a presumably pure growth hormone of their own manufacture which was reported to cause an increase in liver fat content in hypophysectomized as well as in intact rats.

15. Weil, R., and Ross, S., *Endocrinology,* 1949, v45, 207.

16. Li, C. H., Simpson, M. E., and Evans, H. M., *Arch. Biochem.,* 1949, v23, 51.

14. Levin, L., and Farber, R. K., unpublished data.

This is substantiated by other data to be published elsewhere. However, it now becomes evident why certain ACTH preparations are quite active, of themselves, in causing fat mobilization in intact (but not in adrenalectomized) mice. If, as is indicated by the presently reported data, the two factors required to produce this response are an unidentified pituitary substance and 11-oxygenated steroid, then it is apparent that ACTH, slightly contaminated with an impurity of as yet unknown identity, can furnish both factors. The pituitary factor is thus supplied directly from exogenous sources while adrenocortical hormone is caused to be produced endogenously as a result of stimulation of the animal's own adrenals. An alternative possibility is that several pituitary hormones, including ACTH and growth hormone, are able to cause fat mobilization by a *direct action* but that ACTH, by virtue of its ability to cause secretion of adrenal steroids, is able to cause fat mobilization in the intact animal without the need for exogenously supplied 11-oxygenated steroid. Growth hormone, on the other hand, not being able to elicit increased adrenal cortical secretion would accordingly be inactive, even in intact animals, unless appropriate exogenous adrenocortical hormone is supplied.

Summary and conclusions. 1. Many, but not all, pituitary preparations produce an increase in liver fat content when administered to intact mice. This response is completely abolished by adrenalectomy.

2. Neither cortisone nor any other adrenocortical steroid tested to date is able, of itself, to cause a substantial increase in liver fat level of intact or of adrenalectomized mice. In the latter, however, the usual post-adrenalectomy loss of liver fat is prevented.

3. When pre-treated with adrenocortical hormone (cortisone), the ability of adrenalectomized mice to respond to pituitary hormone by mobilization of fat to the liver is equal to or greater than that of intact mice. Pituitary substances (growth hormone; weakly potent ACTH) inactive in either intact or adrenalectomized animals, became quite effective in mobilizing fat to the liver if the animals are pre-treated with adrenocortical hormone.

4. It is concluded that to mobilize fat from the depots to the liver the animal requires at least two factors, (1) adequate supplies of adrenocortical hormone (from either endogenous or exogenous sources) plus (2) a supply, either from its own anterior hypophysis or from exogenous sources, of the "triggering" pituitary factor.

5. The "triggering" action of the unidentified pituitary factor is not mediated via the adrenal cortex since it operates in the absence of the adrenal gland provided adequate amounts of exogenous cortisone are administered.

6. The possible identity of the "triggering" pituitary factor is discussed.

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Possible Usefulness of Substituted Amino Acids for Tumor Growth Inhibition.*† (18040)

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The avidity of transplanted sarcoma in rats for labelled tyrosine(1) and rat hepatoma for labelled alanine and glycine(2) suggested the possibility that amino acids containing a toxic substituent might prove useful for the inhibition of tumor growth. Although N-acetylation of tryptophane does not prevent its utilization as a growth promoter(3). N-iodoacetyl derivatives of L-tryptophane, L-leucine and D,L-phenylalanine were considered suitable for an exploratory study because the iodoacetyl group, an inhibitor of certain respiratory enzymes, is a toxic residue. Accordingly, these derivatives were prepared from corresponding chloroacetyl derivatives by treatment with sodium iodide in acetone(4). For preparation of the radioactive analogues NaI^{131} was substituted for sodium iodide(4). For assessment of their distribution in tissues, their general toxicity and their specific toxicity for tumor tissue, control observations with iodoacetamide were necessary. Radioactive iodoacetamide was similarly prepared from chloroacetamide(2).

The distribution of radioactivity in tumor, liver and blood of Swiss mice bearing transplanted sarcoma 37 was determined at intervals after injection of the radio-active analogues of the three amino acid derivatives and of iodoacetamide. With the possible exception of radioactive iodoacetamide in tumor (Fig. 2, 3, 4) there were no consistently significant differences among the 4 compounds in the concentrations of radioactivity in liver, blood and tumor. Although the concentration of radioactivity in blood and tissues in the initial stages is not significantly different after administration of these 4 compounds than it is after the administration of NaI^{131}, the radioactivity from the latter disappears within 4 hours(5) while from the former small but significant amounts of radioactivity persist for considerably longer periods. This persistence may reasonably be explained by the assumption that at least a small fraction of radioactive iodoacetamide and iodoacetylamino acid derivatives had reached tissue cells.

Significant differences in systemic toxicity of the 4 compounds were observed (Fig. 1). At the LD_{50} on a molar basis, iodoacetamide was more toxic than iodoacetyltryptophane and about twice as toxic as iodoacetylleucine and iodoacetylphenylalanine.

The effect on the growth of the sarcoma 37 in Swiss mice was studied by tumor assay (6). The compounds were administered intraperitoneally in aqueous solution in ten daily doses in groups of from 15-25 mice bearing 3-day-old sarcoma 37. Control animals received saline in equivalent volume. On the 14th day, the tumors were excised and weighed. Two sets of experiments were performed. In the first experiment, in which the

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† This investigation was aided by a research grant from the National Cancer Institute, National Institute of Health, Public Health Service.

‡ Kirstein Fellow in Surgery, Beth Israel Hospital, Boston.

§ We are indebted to Mr. Myron Milden for technical assistance.

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5. Seligman, A. M., Rutenburg, A. M., and Friedman, O. M., *J. Nat. Cancer Inst.*, 1949, v9, 261.

6. Seligman, A. M., Milden, M., and Friedman, O. M., *Cancer*, 1949, v2, 701.

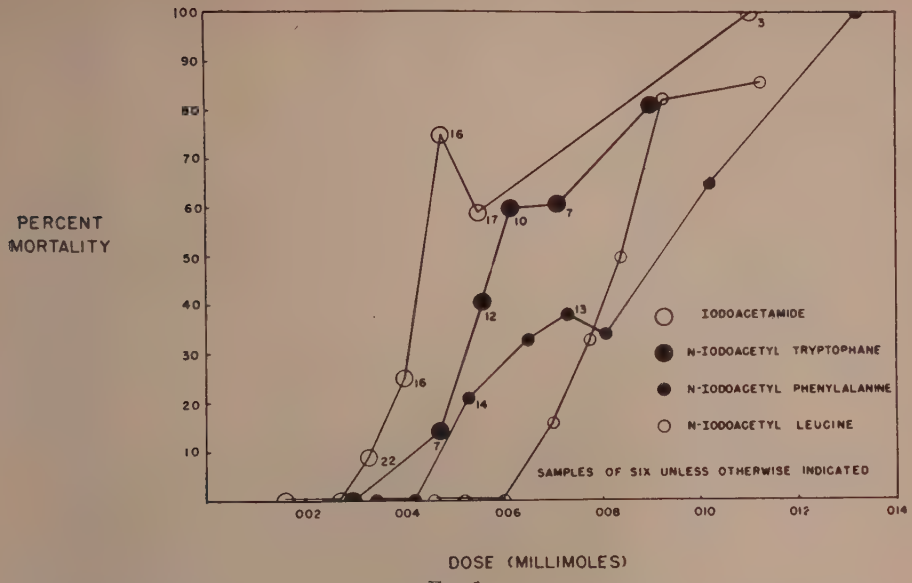

FIG. 1.

Toxicity in Swiss mice after intraperitoneal injection of compounds in saline solution. Bicarbonate used with amino acid derivatives. Per cent mortality based on 10-day survival.

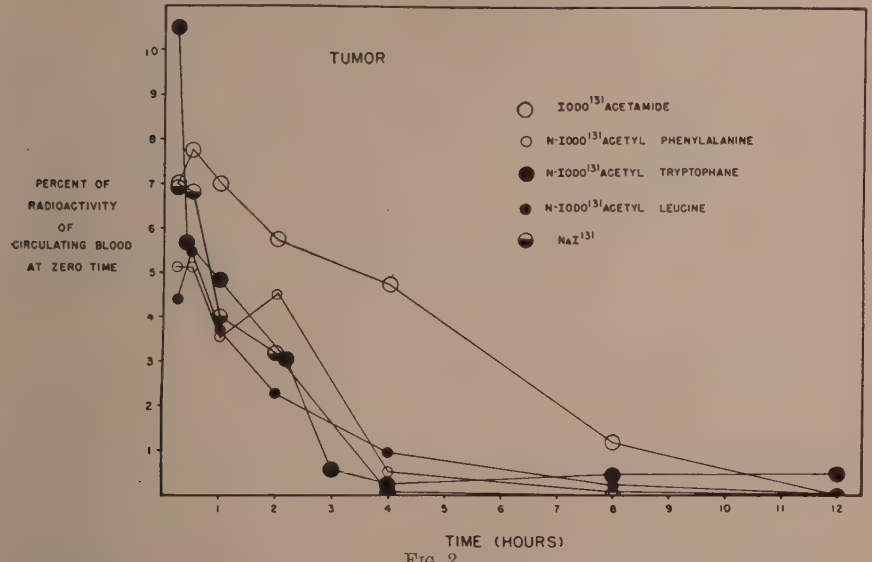

FIG. 2.

Sarcoma 37 in Swiss mice. Compounds administered intravenously in saline solution, bicarbonate being used with amino acid derivatives. Curves represent the average of 2 experiments.

average tumor weight of the untreated mice was 0.88 g the inhibition ratios (*i.e.* the ratio of the mean tumor weight of the untreated to that of the treated mice) was 1.6 for iodoacetylphenylalanine, 1.4 for iodoacetamide, 1.3 for iodoacetylleucine and 1.1 for iodoacetyltryptophane. Even the highest of these ratios is not more than of borderline sig-

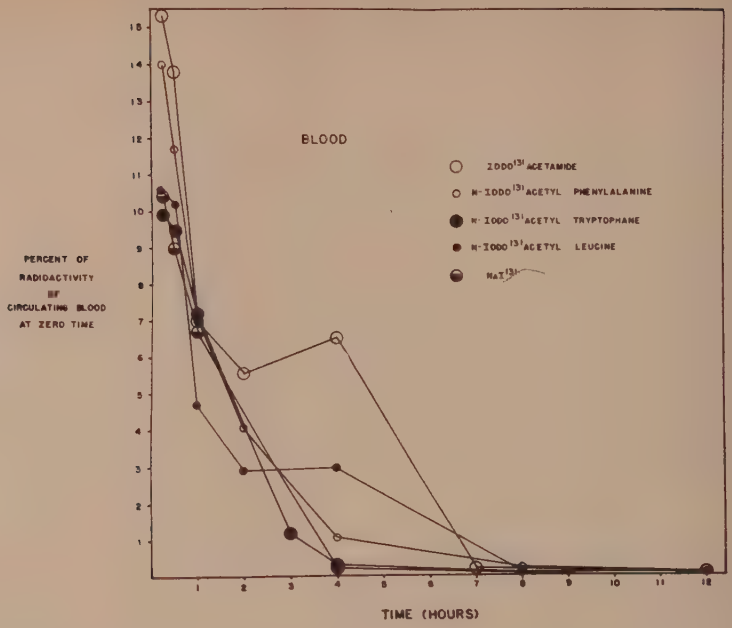

FIG. 3.
Swiss mice bearing sarcoma 37. Compounds administered intravenously in saline solution, bicarbonate being used with amino acid derivatives.

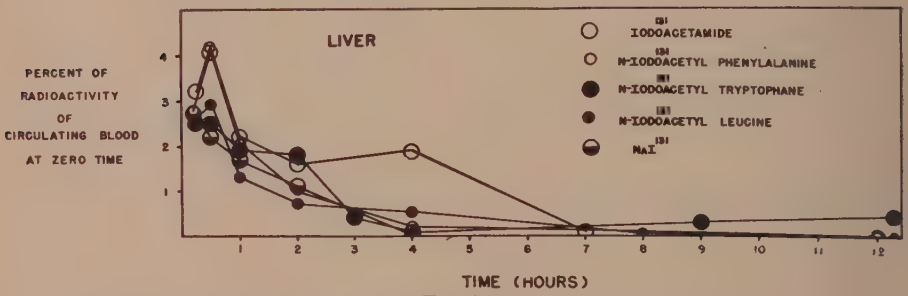

FIG. 4.
Swiss mice bearing sarcoma 37. Compounds administered intravenously in saline solution, bicarbonate being used with amino acid derivatives.

nificance. In the second experiment, however, the average tumor weight of the untreated mice was 1.75 g, the inhibition ratios were 5.2 for iodoacetylphenylalanine, 3.7 for iodoacetamide, 1.9 for iodoacetylleucine and 1.6 for iodoacetyltryptophane. The first two of these ratios are statistically significant. Although these results are not conclusive with regard to the use of these compounds therapeutically, because of limitations inherent in this type of bioassay(6), they do indicate that the tumor growth inhibitory effect of these compounds bears no quantitative relationship to their systemic toxicity. Possibly the nature of the particular derivative rather than the common iodoacetyl radical alone is responsible for whatever inhibitory action is produced. Further investigation of the phenylalanine derivative, therefore, might be justified.

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Some Factors Influencing Enzymic Activities of *Corynebacterium creatinovorans.* (18041)

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Introduction. Dubos and Miller(1) isolated a soil bacterium, later named *Corynebacterium creatinovorans* (Strain "NC"), which could be adapted to grow on creatinine as its sole source of carbon and nitrogen. Krebs and Eggleston(2) found that the enzymes in this organism responsible for the oxidation of creatinine, lactose and malic acid were totally adaptive (according to Karström) (3), whereas those responsible for the oxidation of uric acid, amino acids and lactic acid were partly adaptive and the enzymes responsible for the oxidation of glucose or glycerol were constitutive. As a result of work with this organism on the oxidation of acetate (4) and the adaptive nature of its acetate oxidation system(5), we became interested in some of its other metabolic activities, some results of which are presented here.

Methods. Corynebacterium creatinovorans was grown in media consisting of a carbon source (either glucose, glycerol or acetate), 0.8%; $(NH_4)_2SO_4$ or NH_4Cl, 0.2%; NaCl, 0.4-0.5%; yeast extract, 0.1%; phosphate buffer, pH 7.0, 0.005 M, plus minerals in the following amounts per liter of medium: 3% $MgSO_4$, 1.5 ml; 1% $CaCl_2$, 5 ml; 1% Na_2CO_3, 4 ml; 1% $NaHCO_3$, 2 ml; 0.1% $MnSO_4$, 1 ml; 0.01% HNO_3, 1 ml; 0.1% $FeCl_3$, 0.3 ml. The medium was aerated, and after 24 hours' growth at approximately 28°C, the cells were harvested, washed twice and finally suspended in distilled water. The yield of cells was generally about 4 g, wet weight, per 2 liters of medium. Dry weight determinations were obtained on all cell suspensions. Standard Warburg technic was used, all reaction vessels being buffered with 0.01 M phosphate buffer at pH 7.0, with KOH in the alkali well. The results are expressed as Q_{O_2}, or μl O_2/mg dry weight of cells/hour. Blank Q_{O_2} values (usually 8-16) have been subtracted.

Results. Following the recommendations of Karström(3) and Gale(6) that an adaptive enzyme is one whose production is markedly increased (at least 5-fold), and a constitutive enzyme is one whose production is not significantly increased (less than doubled) by the presence of the *specific substrate* during growth, it appears from the data in Table I that the acetate oxidation enzyme system is adaptive, the glycerol-oxidizing enzyme is semi-adaptive and the glucose-oxidation enzyme system is truly constitutive.

The maximum activity of a culture is

TABLE I.
Activity of Cells Grown in Different Media.

Substrate	Cells grown on: Glucose Q_{O_2}	Glycerol Q_{O_2}	Acetate Q_{O_2}	Nutrient agar Q_{O_2}
Glucose	60	33.9	33.3	41.8
Glycerol	62.8	218.8	66.5	61.5
Pyruvate	12.9	12.1	88.8	28.8
Lactate	18.3		31.0	39.6
Butyrate	6.9	5.2	21.8	
Acetate	53.2	37.2	203.3	
Glycolate			5.5	
Glyoxalate	4.5	3.0	24.0	
Oxalate			0	
Glycine	15.8	22.3	67.3	65.2
Citrate			6.8	
α-ketoglutarate	20.6	18.1	8.0	
Succinate			2.5	
Fumarate	17.1	6.4	11.5	12.1
Malate	10.2	6.9	27.8	16.2
Mg (dry wt) of cells per vessel	4.04	4.04	4.00	4.07

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2. Krebs, H. A., and Eggleston, L. V., *Enzymologia*, 1939, v7, 310.

3. Karström, H., *Ergeb. Enzymforsch.*, 1938, v7, 350.

4. Kalnitsky, G., and Barron, E. S. G., *J. Biol. Chem.*, 1947, v170, 83.

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6. Gale, E. F., *Bact. Rev.*, 1943, v7, 139.

TABLE II.
Activity of Cells Grown for 1-3 Days in Acetate Medium.

Substrate	Cells harvested after 1 day Q_{O_2}	2 days Q_{O_2}	2.5 days Q_{O_2}	3 days Q_{O_2}
Glucose	33.3	38.6	40.9	15.4
Glycerol	66.5	101.5	116.1	64.7
Pyruvate	88.8	38.2	23.5	13.6
Lactate	31.0	19.8	21.4	13.1
Butyrate	21.8	15.6	9.6	8.6
Acetate	203.3	100.3	74.6	30.4
Glycolate	5.5	13.4		2.5
Glyoxalate	24.0	23.1		13.4
Oxalate	0	6.2		0
Glycine	67.3			
Citrate	6.8	8.9		4.0
α-ketoglutarate	8.0	5.9		14.6
Succinate	2.5	5.0		4.0
Fumarate	11.5	1.0		1.0
Malate	27.8	27.8	30.5	14.6
Mg (dry wt) cells per vessel	4.00	4.04	3.84	3.96

usually shown after 18-24 hours growth(6). With this organism, however, the activity with some substrates was greater after 48-60 hours growth (Table II).

TABLE III.
Lag in Acetate Oxidation by Cells Harvested After 2 Days.

Time, hr	Activity of cells harvested after 2 days Q_{O_2}	2.5 days Q_{O_2}	3 days Q_{O_2}
1st	100.3	74.7	30.4
2nd	120.7	139.8	49.8
3rd		147.0	54.2

The activity on different substrates depends not only on the growth medium and the age of the culture, but also on the duration of the test (Table III).

These results would indicate the need for similar studies with other organisms and raises the question as to what might be found during the logarithmic phase of growth.

Summary. The variations in metabolic activity of the soil organism, *Corynebacterium creatinovorans* (Strain "NC"), grown in media containing different carbon sources is described.

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An Antichylomicronemic Substance Produced by Heparin Injection.* (18042)

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Heparin *in vivo* abolishes alimentary lipemia. This effect is not obtained when heparin is added to blood *in vitro*(1-3). Perfusion of heparinized lipemic blood through different parts of the body results in the disappearance of the lipemia suggesting that no specific organ is involved(2). The effect observed is due to a change in the physical state of the fat since the total plasma lipid level is not altered during short term experiments. Heparin injection has also been found to increase the rate of fat uptake from the intestine(3). In the present work the possibility that a surface active substance is produced by heparin administration has been explored.

The chemical composition of heparin would not suggest surface activity(4), and surface tension measurements on 8 heparin preparations in aqueous solution (1 mg/ml) showed none was present. Preliminary experiments

* Heparin was kindly provided by Dr. D. W. MacCorquodale of the Abbott Research Laboratories and Dr. L. L. Coleman of the Research Division, The Upjohn Co.

† Atomic Energy Fellow.

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2. Weld, C. B., *Canad. Med. Assn. J.*, 1944, v51, 578; 1946, v54, 71.

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4. Jorpes, J. E., Bostrum, H., and Mutt, V., *J. Biol. Chem.*, 1950, v183, 607.

TABLE I.
Change in Plasma Percent Transmission and Surface Tension After Injection of 50 mg of Heparin. Surface tension measured in dynes/cm.

	% transmission (700 μ)			Surface tension (S.T.)				
		Post-heparin			Post-heparin			
Exp. No.	Pre-heparin	30 sec.	5 min.	Pre-heparin	30 sec.	5 min.	% T. increase	S.T. drop
1*	49.2	69.8	57.9	51.6	50.1	50.6	8.7	1.0
2	33.1	37.3	65.7	56.3	54.5	49.6	32.6	6.7
3	54.3	60.7	81.0	54.6	52.1	46.7	26.7	7.9
4	13.3	37.2	66.1	56.1	45.0	44.8	52.8	11.3
5	67.0	86.0	85.0	52.5	51.8	51.4	18.0	1.1
6	68.0	—	86.8	56.9	—	56.6	18.8	0.3
7	70.0	—	85.2	59.2	—	51.6	15.2	7.6
8	46.2	—	80.2	56.7	—	53.1	34.0	3.6
9	64.2	—	85.9	54.4	—	50.8	21.7	3.6
10	76.2	—	87.1	53.9	—	51.8	10.9	2.1
11	76.2	—	83.9	53.7	—	51.7	7.7	2.0
12	75.0	—	86.0	58.2	—	54.1	11.0	4.1

* Only 25 mg heparin inj. in this instance.

in the dog and the human with preservative free heparin solutions demonstrated that the antilipemic effect is due to heparin itself, and not to the preservative (usually phenol). The remaining experiments were performed with heparin solutions as supplied commercially.

The effect of heparin injection on plasma absorption spectrum and surface tension was studied in twelve experiments in healthy human adults. Three to 4 hours after a fatty meal a blood sample was obtained by venipuncture. Heparin was then introduced through the same needle. Additional blood samples were obtained at 30-second and 5-minute intervals after the heparin was injected. In some instances only one post-heparin sample was taken at 5 minutes. All blood samples were citrated and centrifuged at once. Control experiments with defibrinated blood demonstrated that citrate has no adverse effect. In one instance 25 mg of heparin was administered; in the rest a standard dose of 50 mg was used. The optical transmission of the plasma over the range 400 mμ to 700 mμ was determined in all instances. Optical measurements were made in 10 x 75 mm round cuvettes in a Coleman Junior Spectrophotometer. Surface tension measurements were made on undiluted plasma with the duNoüy tensiometer using the special precautions recommended(5).

5. duNoüy, P. L., *Surface Equilibria of Biological and Organic Colloids*, N.Y., 1926.

The results are shown in Table I. The surface tension drop varied from 0.3-11.3 dynes/cm after heparin injection, and the percent transmission rise varied from 7.7-52.8% at 700 mμ. In the instances where cholesterol was followed, no significant change was observed. The detailed results of one experiment are shown in Fig. 1. It is evident that a decrease in surface tension accompanies the heparin-induced clearing of lipemic plasma. This suggests that a surface active agent is formed when heparin is injected. In fact, the presence of this antilipemic substance can be demonstrated *in vitro* by the loss of turbidity occurring when lipemic pre-heparin plasma and cleared post-heparin plasma are mixed. An experiment demonstrating this is shown in Fig. 2. It should be noted that little change in optical density takes place in the plasmas from which the mixture was made. The antilipemic surface active substance, though formed only *in vivo*, clears lipemic plasma both *in vivo* and *in vitro*.

Contrary to previous reports(3) the turbidity did not return to heparin-cleared plasmas left 24 hours at room temperature. Instead continued clearing was noted. The difference between pre- and post-heparin plasmas is more marked if the samples are refrigerated since the low temperature causes the lipemic plasma to become more opaque.

Alcohol extracts of heparin cleared and

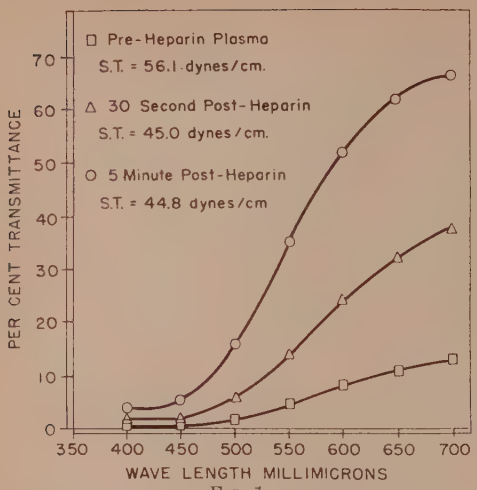

FIG. 1.

Effect of injecting 50 mg of heparin intravenously on plasma absorption spectrum and surface tension (S.T.).

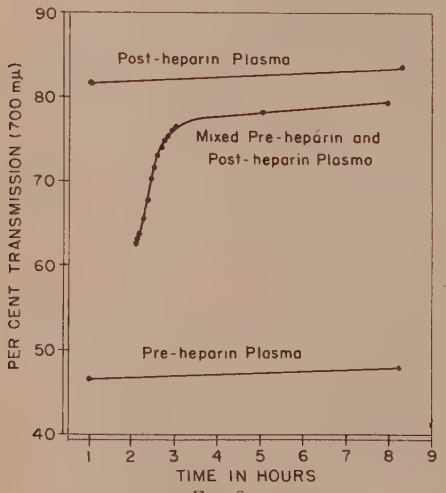

FIG. 2.

Effect of mixing pre-heparin lipemic serum with cleared serum obtained from the same individual 5 minutes after injecting 50 mg of heparin intravenously.

uncleared plasmas showing little difference in surface tension were evaporated to dryness and then shaken up in water. The post-heparin preparations had a surface tension about 10 dynes/cm lower than the corresponding pre-heparin plasma preparations. More striking than the surface tension drop was the soapy nature of the post-heparin preparation demonstrated by the formation of a persistent foam. Since plasma proteins were removed in making these preparations, protein would not seem to be a constituent of the surface active material present.

n-octylamine heparinate, a slightly soluble heparin salt(6), exhibits considerable surface activity in very dilute solutions. While this observation is suggestive, the surface activity of n-octylamine chloride itself makes experimental work with the heparin salt inconclusive. The surface activity of heparin in combination with choline containing lipids was therefore studied.

When heparin was added to a colloidal egg lecithin solution no decrease in turbidity was noted. In agreement with previous findings(7) a solution containing 5.1 mg/ml of fresh egg lecithin was found to have essentially the same surface tension as water. The addition of heparin did not alter the surface tension. When heparin and lecithin were precipitated together from an alcohol solution by acetone a surface active material was obtained which gave a low surface tension in water and formed a soapy solution. The lecithin used in these experiments was freshly prepared and was reprecipitated with acetone four times. However, it is not sufficiently pure to exclude the possibility that other substances may be responsible for this effect. The failure of heparin to form a surface active combination with lecithin when mixed with it in water may be due to the presence of impurities or to the colloidal nature of the lecithin under these circumstances—a factor which may also prevent heparin from forming a complex with the basic phospholipids of lipemic blood *in vitro*. Whether heparin combines only with oriented phospholipids of the cell surfaces lining the vascular system, combines with blood phospholipids only under conditions existing in capillary beds, or is enzymatically combined with phospholipids, cannot be profitably con-

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sidered at this time.

It should be noted that when the aqueous lecithin solution used above was allowed to stand 2 weeks it became highly surface active. Whether this change is correlated with the oxidation of the lecithin or not is not known.

Discussion. Measurement of the surface tension reducing activity of a substance is not a true indication of its emulsifying and detergent properties. This is especially true when measurements are made in the presence of a large amount of fat as is the case here. However, such measurements constitute the only direct approach available. The fact that any surface tension lowering of plasma is observed is surprising since the surface active material might be expected to follow the fat and not accumulate at the air-water interface.

The surface activity ascribed to lecithin is believed to be due to the presence of polysaccharide impurities(7) since solutions of pure lecithin show little surface activity. Commercial lecithin preparations contain large amounts of carbohydrate some of which cannot be removed(8). Lecithin has also been reported to occur in loose combination with carbohydrate in plants(9). MacLean(10) in attempting to fractionate a phospholipid preparation of animal origin discovered a polysaccharide derivative which we know as heparin. The findings reported in this paper are therefore suggested to some extent by the initial work of MacLean, and the association of phospholipids with polysaccharide derivatives such as heparin may be a very general phenomena.

The surface active agent responsible for the clearing of lipemic plasma *in vivo* and *in vitro* may be a heparin-phospholipid complex. The highly acidic heparin molecule may possibly be attached to the extremely basic choline of a phospholipid similar to lecithin, which has a theoretical isoelectric point of 7.5(11). The effects of choline deficiency on fat transport, insulin sensitivity, and liver fats are well known(12) and may be due in part to a failure to produce the surface active substance described here. The possible relationship of the findings reported here to pathological conditions related to chylomicronemia such as atherosclerosis(13) and the lipemia occurring in diabetes and acute pancreatitis deserve special attention.

In view of the deleterious effects of intravenous fat injections due to the size of the fat droplets and the uptake of fat by the reticulo-endothelial system(14), the use of heparin and heparin-phospholipid complexes for therapeutic fat administration is being investigated.

Except in instances where normal blood heparin levels exist, the use of chylomicron counts to follow fat absorption is questioned.

The surface active, chylomicron dissolving effect demonstrated has been tentatively termed achylin activity.

Summary. The injection of heparin (50 mg intravenous) in the human causes a sudden fall in surface tension and a clearing of the lipemic plasma. The effect is believed to be due to the formation of a surface active heparin-phospholipid complex. The activity of the complex may be studied *in vitro* by mixing lipemic plasma with non-turbid plasma obtained after heparin injection. A gradual clearing is observed. The formation of a surface active heparin-egg lecithin complex has been demonstrated. Certain relations of these findings to pathological conditions are discussed.

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'Benemid', p-(di-n-propylsulfamyl)-benzoic Acid: Inhibition of Glycine Conjugative Reactions.* (18043)

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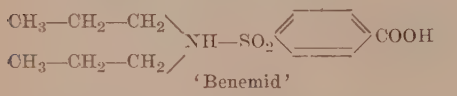
The purpose of this report is to present in general terms what seems to be a common enzymological basis for a number of physiological phenomena that have appeared to be unrelated. From this research has been developed a new compound, 'Benemid'†, p-(di-n-propylsulfamyl)-benzoic acid,



'Benemid'

synthesized by Miller, Ziegler and Sprague (1), that has interesting fundamental characteristics, and for which a diversification of clinical inquiry seems indicated.

From an analysis of the enzymic factors in the secretion of phenol red by the mammalian renal tubule *in vitro*, Beyer, Painter and Wiebelhaus(2) found that it was possible to block the utilization of energy from the phosphate cycle for phenol red secretion without impairing oxidative or transphosphorylative processes or decreasing the utilization of energy from phosphorolysis of energy-rich phosphates (*e.g.*, adenosinetriphosphate, ATP) for other essential functions of the cell or for the body as a whole. Thus it was concluded that inhibitors of this latter type blocked the ability of a definitive enzyme or enzyme system to utilize energy from phosphorolysis of energy-rich phosphates (~Ph) for a (conjugative) reaction that is essential for the ultimate secretion of phenol red. Insofar as they are comparable, these findings are in agreement with the recent similar studies of Taggart and his associates(3) relating coupled oxidation-phosphorylation reactions to phenol red secretion.

Concurrently, we have studied the characteristics of the conjugative reaction involved in the formation of p-aminohippuric acid (PAH) from p-aminobenzoic acid (PAB) and glycine, described by Cohen and McGilvery (4). They found that the conjugase required a coupled source of energy for the completion of that conjugation in the kidney or liver. A parallelism between the requirements of high energy phosphate bonds for both synthesis and secretion of PAH (which is secreted by the same renal tubular mechanism as is phenol red) has been suggested elsewhere(5).

'Benemid' has been found to be an interesting example of a class of potent inhibitors of the conjugation reaction leading to PAH synthesis. This reaction is not peculiar to PAH or hippurate formation *per se*, but it relates to the conjugation likewise of other compounds, such as p-aminosalicylate, with glycine. Inhibitors of the conjugative inactivation of endogenous or therapeutically useful agents have been referred to tentatively as anticatabolites(6).

Procedure. The system and methods of Cohen and McGilvery(4) were employed for studying the conjugation of PAB with gly-

* Presented in part before the Pharmacological Section, Fed. of Biol. Sciences, April, 1950(6).

† 'Benemid' is the trademark that has been applied to p-(di-n-propylsulfamyl)-benzoic acid by Sharp & Dohme, Inc.

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5. Beyer, K. H., *Pharmacol. Rev.*, 1950, v2, 227.

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cine. The Warburg respirometer apparatus was employed, the flasks and their contents being shaken at 37°C for a period of one hour before analyzing for PAB and PAH, unless otherwise indicated in the text. The flasks contained:

Washed liver or renal cortex residue	0.6 ml
PAB, 0.02 M	0.2
Glycine, 0.15 M	0.5
Succinate, 0.154 M	0.3
Cytochrome C, 2 × 10-4 M	0.2
KCl, 0.5 M	0.4
$MgSO_4$, 0.154 M	0.1
K_2HPO_4, 0.3 M, pH 7.6	0.6
Adenylic acid, 0.0164 M	0.1
H_2O, with or without inhibitor added to make a total volume of 4.0 ml	

Guinea pig liver or renal cortex was homogenized in cold KCl and was washed 3 times by centrifugation. The final residue was suspended in cold isotonic KCl solution to contain 8 to 11 mg of nitrogen per ml of suspension.

This system regularly yielded a 90% or greater conjugation of PAB (4 μ moles/flask) when incubated aerobically for one hour. The observations of Cohen and McGilvery (4) with respect to the need for adenylic acid, inorganic phosphate, aerobiosis and the presence of a suitable oxidizable substrate have been confirmed. ATP, but not adenylic acid, will provide the necessary energy for the conjugation in the absence of aerobiosis.

Results. Fig. 1 illustrates the relationship of the molar concentration of 'Benemid' to the inhibition of PAB conjugation. Here it may be seen that, in the absence of 'Benemid,'

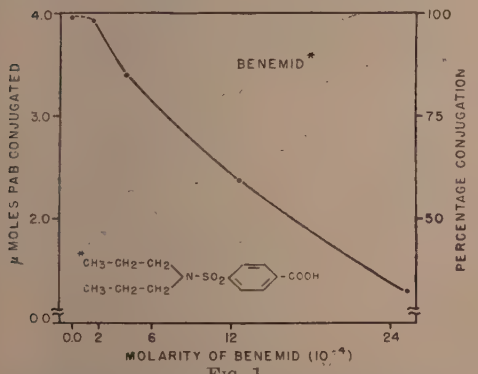

FIG. 1.

Relationship between molar concentration of 'Benemid' and extent of inhibition of p-aminobenzoic acid conjugation with glycine.

conjugation of PAB with glycine was essentially complete. The inhibitory effect of 'Benemid' on the conjugative reaction occurred without an effect on the oxygen uptake of the preparation. Likewise, it can be demonstrated that the conjugation of p-aminosalicylate (PAS) with glycine to form p-aminosalicylurate (PASU) is inhibited by 'Benemid.' However, since the conversion of PAS to PASU is not complete under these conditions it is being studied further.

'Benemid' is equally effective in the ATP-anaerobic system as an inhibitor of PAB conjugation, as is illustrated in Table I. In these experiments the duration of incubation of PAB with glycine was 30 minutes instead of one hour. Therefore, it appears that the inhibitor acts at some point beyond the oxidation/phosphorylation, or ~Ph generating, stage.

'Benemid' has no effect on the *in vitro* phosphorylation of glucose, Table II, at all concentrations of the inhibitor that have been studied (8.3×10^{-4} M to 5×10^{-3} M). The manometric method of Colowick and Kalckar (7) was employed for these studies. For each mole of phosphate transferred from ATP to glucose one acid equivalent is formed which displaces one equivalent of CO_2 from the bicarbonate buffer. Iodoacetate was used to stop the glycolytic reactions at the triosephosphate stage. Fluoride was added to inhibit the action of phosphatases. A lyophilized powder of a dialyzed extract of dog brain was prepared in the manner described by Wiebelhaus and Lardy (8) and was used as the source of the hexokinase.

Discussion. From the data presented above and documented in other experiments it is apparent that 'Benemid' is a potent inhibitor of the conjugation of glycine with either PAB or PAS, and that the inhibition involves specifically the utilization of energy by the reversibly inhibited conjugase, rather than by prevention of the production of ~Ph or a generalized inhibition of its utilization. It

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TABLE I.
Inhibition of PAB-Glycine Conjugation* by 'Benemid.'

Adenylic acid, final molarity	ATP, final molarity	Gas phase	'Benemid' final molarity	PAB conjugated, %	Inhibition of conjugation, %
		Exp. 1.			
		Air		1.6	
.0004		N_2		1.4	
.004		N_2		2.2	
.004		Air		68.7	—
.004		Air	.00125	19.8	70.2
.004		Air	.00250	14.4	79.0
	.004	Air		83.2	—
	.004	Air	.00125	37.4	55.2
	.004	Air	.00250	5.6	93.4
		Exp. 2.			
	.006	Air		59.1	
	.006	Air	.00125	53.0	10.2
	.006	Air	.00250	37.7	32.8
	.006	N_2		26.0	
	.006	N_2	.00125	22.2	14.6
	.006	N_2	.00250	18.2	30.0
.006		N_2		1.3	

* Incubated for one-half hour at 37°C. Where ATP was employed, one-half the total amount was tipped in at "0" time. The other half was tipped in from a second sidearm at 15 minutes.

TABE II.
Lack of an Effect of 'Benemid' on Enzymatic Transphosphorylation Between ATP and Glucose.*

Glucose final molarity	ATP final molarity	'Benemid' final molarity	CO_2 liberated μl
	.008		32
.01	.008		398
.01	.008		409
.01	.008	.00083	403
.01	.008	.00083	398
.01	.008	.00167	397
.01	.008	.00167	391
.01	.008	.005	388
.01	.008	.005	391

* For each mole of phosphate transferred from ATP to glucose one acid equivalent is formed and a molar equivalent of CO_2 is displaced from the bicarbonate buffer. Gas phase was 95% N_2 — 5% CO_2 mixture. The system was incubated at 37°C. Duration of the experiment was 30 min.

is our present impression that glycine and perhaps other conjugative mechanisms are implicated fundamentally in certain processes of secretion; the "catabolism" of essential metabolites, possibly some hormones, certain therapeutically useful agents, and in some other as yet ill-defined reactions.

The inhibition of PAB conjugation with glycine by 'Benemid' cannot be considered as an effect on peptide bond formation because of the lack of an α-amino group adjacent to the PAB-carboxyl group that is involved in the amide formation. Obviously, PAH is not a typical dipeptide since the α-amino group is not repeated in the molecule. In long-term chronic toxicity experiments, 'Benemid' had no effect on protein metabolism(9). The disposition of 'Benemid' by the body has been described elsewhere(6,10).

Clinical substantiation of the "anticatabolite" inhibition of PAS (p-aminosalicylate) conjugation with glycine by 'Benemid' has been indicated by Boger, Gallagher and Pitts (11) who reported that the coadministration of 'Benemid' with PAS increased the plasma concentration attained from a given dose of the latter compound. 'Benemid' inhibits the renal tubular secretion of penicillin(5,12), but is itself reabsorbed almost entirely by the tubules rather than being eliminated by

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them(6,12).

Summary. 'Benemid', p-(di-n-propylsulfamyl)-benzoic acid, inhibits the conjugase responsible definitively for the over-all reaction involved in the conjugation of glycine with p-aminobenzoic acid. It was demonstrated that the compound did not decrease the availability of energy derived from the phosphate cycle, which is a coupled component of the conjugative reaction, since 'Benemid' did not inhibit glucose phosphorylation by phosphorylase plus preformed ATP.

The implication of such conjugative reactions was discussed and several pharmacological and clinical applications of the anti-catabolite hypothesis were indicated.

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Labelling in the Glucose Deposited as Starch during Photosynthesis. (18044)

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We have extracted radioactive starch from detached tobacco leaves which were permitted to carry on photosynthesis in an atmosphere of 5% $C^{12}O_2$ containing about 0.25 mc of $C^{14}O_2$. Prior to the experiment plants were kept in darkness to deplete their initial starch reserves. The extracted starch was hydrolized, and on addition of ordinary glucose, as a carrier, radioactive glucose was crystallized. The technic of the experiments was essentially that of Putman *et al.*(1). Radioactive glucose obtained was fermented with *Lactobacillus casei* and degraded according to Wood *et al.*(2). The results are shown in Table I.

From the results of the Experiment 1, it is evident that if after 24 hours of preliminary starvation photosynthesis is permitted to take place for a long time, then C^{14} is spread fairly evenly in all positions.

It has been shown by Benson *et al.*(3) that in the initial stages of photosynthesis C^{14} appears in glucose at first in the 3 and 4 positions and only later spreads to others. It was thought that an extension of the period of darkness prior to photosynthesis may accentuate this tendency, and that under these conditions either the bulk of C^{14}, or even all of it, may appear in the positions 3 and 4. To test this, plants were at first starved for 72 hours and then permitted to carry on photosynthesis for various periods of time. The results are represented by Experiments 2, 3 and 4, Table I.

While the bulk of C^{14} is now in the 3 and 4 positions, considerable amounts still are present in the 2 and 5 and the least in the 1 and 6. The results are essentially the same in all 3 experiments in spite of the fact that the duration of photosynthesis in them varied from 3½ to 48 hours. One can conclude, therefore, that it was the length of the preceding starvation period and not the length of the illumination, which was responsible for the observed distribution of C^{14}.

One may also expect that if a period of photosynthesis in $C^{14}O_2$ is followed by that in $C^{12}O_2$, the final labelling in starch will be reversed. The greatest activity should be in 1 and 6, followed by that in 2 and 5, and the least in 3 and 4. To test this, 2 detached leaves were permitted to carry on photosynthesis for 3 hours in an atmosphere of $C^{14}O_2$. At the end of this period one leaf was killed and the distribution of C^{14} was determined. The other leaf was transferred to 5% $C^{12}O_2$

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TABLE I.
Distribution of C^{14} in Glucose Derived from Starch. % of Total Activity in Various Positions.

			3 and 4 %	2 and 5 %	1 and 6 %
Exp. 1	24	hr of darkness			
	24	" " illumination with $C^{14}O$	34.3	33	32.7
Exp. 2	72	" " darkness			
	3½	" " illumination " "	52	25.9	22.1
Exp. 3	72	" " darkness			
	24	" " illumination " "	47.5	39	13.5
Exp. 4	72	" " darkness			
	48	" " illumination " "	54.5	25.5	20
Exp. 5	16	" " darkness			
	3	" " illumination " "	41	37	32
Exp. 6	16	" " darkness			
	3	" " illumination " "			
		followed by			
	3	" " illumination " $C^{12}O_2$	14.3	32.7	53

and was permitted to carry on photosynthesis for 3 hours longer. The results obtained, as shown in the Experiments 5 and 6, indicate that the distribution of C^{14} was changed as expected.

There is one other point of interest which appeared from the Experiment 5. A short period of illumination of 3 hours, after an overnight darkness of 16 hours resulted in fairly even but not quite uniform distribution of C^{14}. Like in the sugars produced in photosynthesis initially(3) there was somewhat more activity in 3 and 4 than in 2 and 5 and the least in 1 and 6.

From the results of our experiments we can conclude, therefore, that the distribution of C^{14} in glucose, deposited during photosynthesis as starch, may be affected by the following factors: 1. Duration of the starvation period prior to the illumination. 2. Duration of the illumination period itself. 3. By permitting plants to carry on photosynthesis at first in an atmosphere of $C^{14}O_2$ and then in $C^{12}O_2$.

Summary. It is shown that glucose recovered from starch deposited in photosynthesis in presence of $C^{14}O_2$ may be labelled primarily in 3 and 4 or 1 and 6 positions or evenly in all 6 positions, depending upon the periods of darkness and illumination to which the leaf is subjected.

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Effects of X-Irradiation and Urethane Treatment on Chicken Bone Marrow Enzymes.* (18045)

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Previous work(1,2) has shown that the injection of aminopterin into mature hens results in a leucopenia, reduced bone marrow cell counts, and a loss of bone marrow choline oxidase. It was suggested that the loss of marrow choline oxidase may have been a sig-

* Research paper No. 909, journal series, University of Arkansas. This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

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TABLE I.
Effect of X-irradiation and Urethane Treatment on Chicken Bone Marrow Enzymes and on Peripheral Leucocytes.

Treatment	No. of chickens	Oxygen consumption per mg of nitrogen per hr: Endogenous Avg, μl	Endogenous Range, μl	Choline oxidase Avg, μl	Choline oxidase Range, μl	Succinoxidase Avg, μl	Succinoxidase Range, μl	Avg peripheral leucocytes, thousands per μl
Controls	5	16.5	7.2-27.4	3.7	3.0-4.5	9.7	5.6-13.7	29.5
X-ray	5	8.5	6.4-11.2	0.5	0 -1.2	1.0	0 - 2.6	13.1
Urethane	2	16.1	6.0-26.1	1.3	0.6-1.7	5.2	3.5- 6.8	9.4

nificant factor in the leucopenic effect of aminopterin. To investigate further a possible relationship between blood cell formation and marrow choline oxidase, studies have been made on the effects of X-irradiation and urethane treatment on the peripheral leucocytes and bone marrow enzymes of mature hens.

Experimental. Adult hens were exposed to a single whole body irradiation of 300 roentgens (measured on the skin) given over a 32-minute period (375 KV; 5 MA; inherent filtration only; HVL 2.0 mm copper; distance from target to center of animal 123 cm; field size covering entire animal). After exposure hens were killed at intervals of 1, 2, 4, 8, and 17 days. Bone marrow studies were carried out as previously described(2). Since there were no significant differences due to the interval after exposure, only average values and range are given in the table.

One hen was injected intraperitoneally with a total of 5 g of urethane administered at intervals over a 14-day period; another was injected with 1 g of urethane daily for 3 days. The bone marrow was treated as previously described.

The controls were hens of similar size but were given no treatment. All the hens were fed a commercial laying mash.

Results and discussion. The results are presented in Table I. X-irradiation resulted in a marked reduction in endogenous respiration, choline oxidase, and succinoxidase. Urethane injection did not reduce endogenous respiration, but reduced choline oxidase to one-third of control values. Succinoxidase was also reduced by urethane treatment but not as markedly as was choline oxidase. Both urethane and X-rays rendered the birds leucopenic.

The effects of X-irradiation on the enzymes cannot be fully explained. However it has been suggested that X-irradiation inactivates sulfhydryl groups(3) and it is known that both choline oxidase and succinoxidase depend on intact sulfhydryl groups for activity(4,5). The mechanism whereby urethane inhibits choline oxidase is not known; it has been found not to be strictly competitive in nature(6).

Summary. Mature hens were given a single whole body X-irradiation of 300 roentgens. This treatment markedly reduced the bone marrow endogenous respiration, choline oxidase, and succinoxidase. The intraperitoneal injection of urethane also reduced marrow choline oxidase, and reduced succinoxidase but to a lesser degree. Both X-irradiation and urethane treatment rendered the chickens leucopenic.

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Mucolytic Enzyme Systems. XIV. Effect of Certain Quinolines on Hyaluronidase and Its Serum Inhibitor.* (18046)

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The occurrence of the invasive factor, hyaluronidase, in many bacteria is well known. That it might also be employed by animal parasites in their invasive functions is a possibility that has been relatively neglected. However, Levine *et al.*(1) have recently demonstrated that cercariae of *schistosoma mansoni* contain hyaluronidase, and, with the collaboration of Dr. F. G. Wallace of our Zoology Department, an investigation is now underway to determine whether it is present in other parasites as well.

It was observed by Bieter and coworkers(2) that certain 8-amino quinolines exerted a pronounced chemotherapeutic effect in mice infected with *schistosoma mansoni*, while other chemically related compounds were relatively ineffective. The present communication is concerned with an attempt to determine whether the difference in the action of some of these compounds is connected to their effect, *in vitro*, on hyaluronidase or its serum inhibitor.

Materials and methods. The preparation of the hyaluronidase from bull testes followed the procedure used previously(3), and the original method for the preparation of the hyaluronic acid from human umbilical cords was used(3) with the modifications subsequently introduced(4,5). The enzyme activity and its inhibition were determined by the viscosimetric method(3) utilizing those changes in detail that were later employed(5).

The reaction mixture for studies of the effect of compounds on the hyaluronidase was prepared by adding 1.5 cc of the aqueous solution of the compound to be tested to 0.5 cc of the borate buffered enzyme, allowing to stand 6-10 min at 38°C, and then mixing with 4.0 cc of the veronal buffered substrate. 5.0 cc was pipetted into the viscosity tube for measurements at 38°C. The reaction mixture used for studies of the effect of compounds on the serum inhibitor of hyaluronidase was made up in a similar manner; the only difference being that normal human blood serum was first added to the solution of the compound to be tested so that 1.5 cc of the solution contained 0.04 cc of serum. When serum alone was used as the inhibitor, 0.04 cc of it was diluted with distilled water to 1.5 cc for addition to the 0.5 cc of buffered enzyme. The results were expressed as percent inhibition of the hyaluronidase activity(4).

Results and discussion. Two compounds, rosaniline and plasmochin, that had little chemotherapeutic effect in mice infected with *schistosoma mansoni*, were compared with two chemically related effective compounds, 6-methoxy-8-(β-diisobutylaminomethyl) amino-quinoline dihydrochloride (SN 2842) and 6-methoxy-8-(diisobutylaminoisopropyl) amino-quinoline dihydrochloride (SN 3501). However, of the above compounds, only rosan-

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† The authors are grateful for the technical assistance of Mrs. H. Kaufmann and Mr. P. Edmondson.

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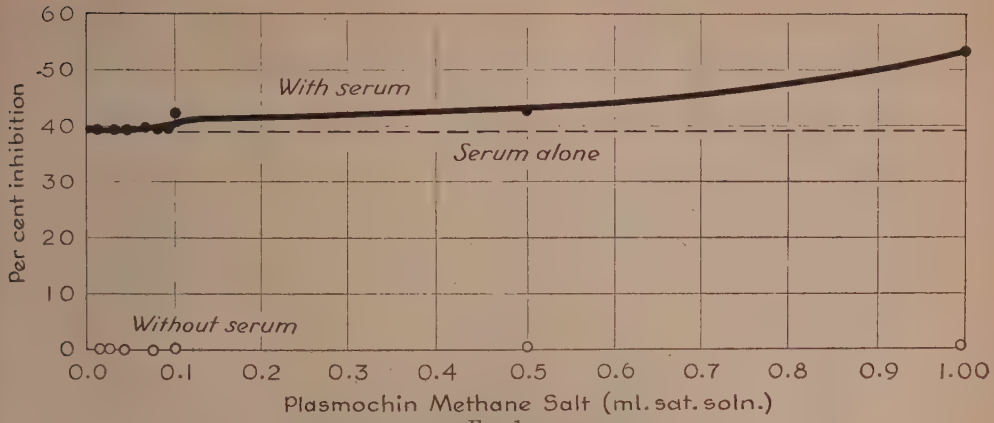

FIG. 1.
Effect of plasmochin on hyaluronidase and its serum inhibitor.

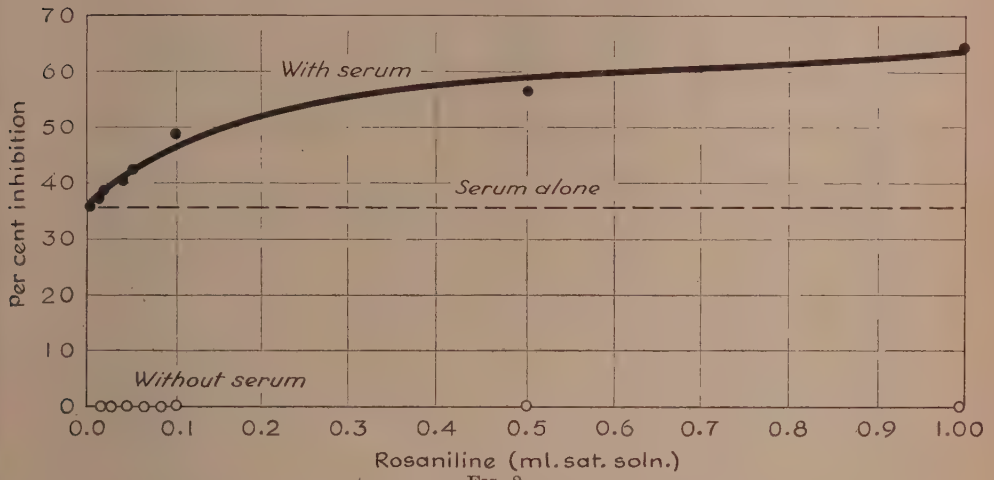

FIG. 2.
Effect of rosaniline on hyaluronidase and its serum inhibitor.

iline was found to exert a prophylactic effect in the infection. To produce this effect the compound must be administered daily for several weeks before, or within the first 2 weeks after, infection. The results are given in Fig. 1-3. The abscissae in all figures refer to quantity of drug per cc final reaction mixture.

It is apparent that, at the concentrations used, neither plasmochin nor rosaniline, Fig. 1-2, had a demonstrable direct effect on the hyaluronidase. Both exhibited some potentiation of the serum inhibitor, the influence being more pronounced with rosaniline. In both instances it will be noted that saturated (25°C) aqueous solutions of the drugs were employed; this was necessitated by the relative insolubility of the substances.

In contrast to these findings, the two compounds chemotherapeutically effective were potent inhibitors of the hyaluronidase at concentrations greater than 0.32 mg per cc reaction mixture; complete inhibition was attained at 1.28 mg per cc, Fig. 3. It may also be seen that these two substances inactivate the naturally occurring serum inhibitor at the same time they exert their direct inhibition on the hyaluronidase. The

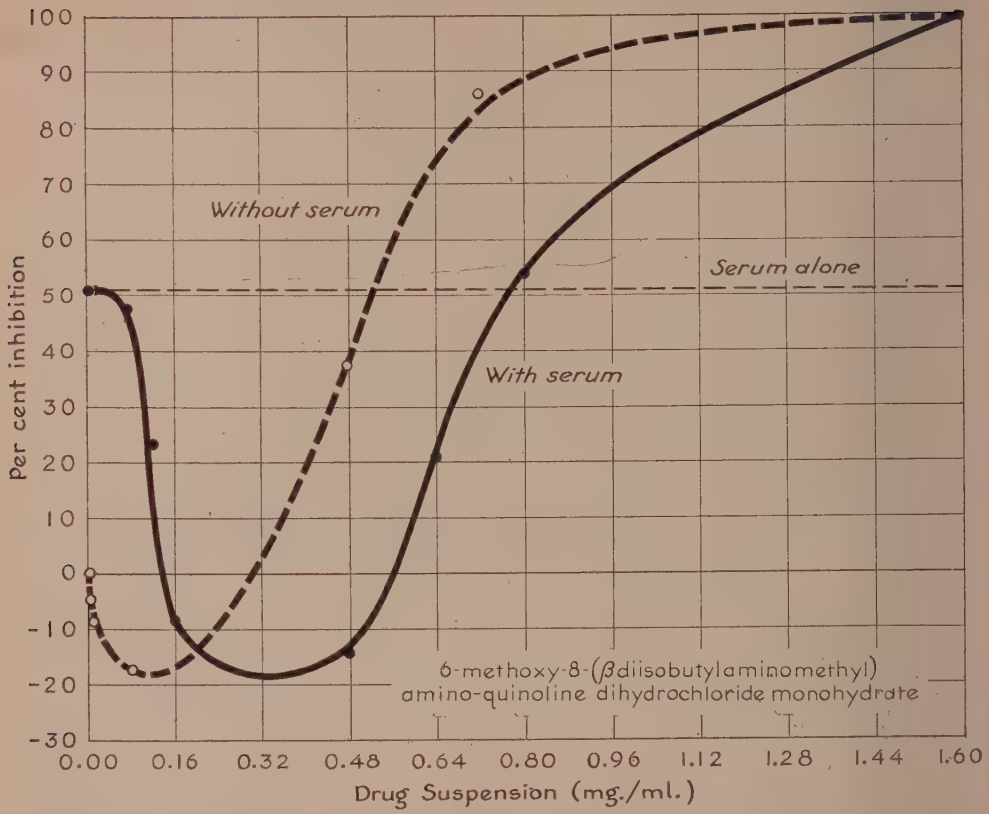

FIG. 3.

Effect of 6-methoxy-8-(β-diisobutylaminomethyl) amino-quinoline dihydrochloride monohydrate on hyaluronidase and its serum inhibitor. Curves practically identical with these were obtained for 6-methoxy-8-(diisobutylaminoisopropyl) amino-quinoline dihydrochloride.

inactivation of the serum inhibitor is manifest at the lowest concentrations of the drugs, and this effect is overtaken by the direct inhibition of the enzyme by the compounds at higher concentrations. Thus, with or without the presence of the serum inhibitor, the drugs produce a complete cessation of the enzyme activity at 1.60 mg per cc. No explanation can be offered at present for the slight degree of activation of the hyaluronidase in the absence of serum that was observed at the lowest drug concentrations.

Summary. Two compounds, 6-methoxy-8-(β-diisobutylaminomethyl) aminoquinoline dihydrochloride and 6-methoxy-8-(diisobutylaminoisopropyl) aminoquinoline dihydrochloride, which are chemotherapeutically effective in mice infected with *schistosoma mansoni,* have been shown to be potent inhibitors of hyaluronidase although they inactivate the serum inhibitor of the enzyme. Chemically related rosaniline and plasmochin, that have little chemotherapeutic effect, had no demonstrable direct effect on the hyaluronidase, although both potentiated the serum inhibitor to some degree.

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Age and Sex Differences in Weight of Pituitary Gland in Dogs. (18047)

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Absolute and relative hypophysis weight is greater in the female than in the male adult human(1,2), rat(3,4) and pigeon(5). This difference does not exist before puberty in the human(6), rat(7), and rabbit(8). Indeed, Allanson(9) found no sex difference in either immature or adult rabbits, while in the woodchuck(10) the hypophysis is apparently larger in the male.

The present investigation was undertaken in order to determine the hypophysis weight-body weight (HW/BW) ratios in immature, adult, and aged dogs.

Methods. The dogs in which these ratios were determined had been used for acute studies in intestinal absorption. It was felt that these experiments did not affect the hypophysis.

The dogs weighed from 3.71 to 35.5 kg (mean, 8.89 kg). Fifty-two of the total of 91 dogs weighed from 5 to 9 kg. They were grouped by body weight in order to compare animals of similar size (Table I). While the exact age was not known, dogs whose teeth and genitalia indicated unmistakably that they were immature were classified as puppies.

TABLE I.
Hypophysis Weight-Body Weight Ratios (mg per 100 g body weight) in Dogs.

Group	No. of animals	HW/BW	p*
All puppies	16	86.7	
" adults	75	73.4	0.017
Adult females	45	76.6	
" males	30	67.0	0.014
Female puppies	7	90.6	
Male puppies	9	83.7	1.0
Adults, both sexes, 3.71-7.00 kg	27	80.9	
Same, 10.01-35.5 kg	19	59.7	<0.01
Adults, both sexes, 7.01-10.00 kg	29	75.4	
Same, 10.01-35.5 kg	19	59.7	<0.01
Adults, both sexes, 3.71-7.00 kg	27	80.9	
Same, 7.01-10.0 kg	29	75.4	0.3
Senile	3	49.6	
All adults	75	73.4	0.02

* "p" is probability of chance difference between two means. For statistical significance, "p" must be 0.05 (1 chance in 20) or less(14).

The calvaria was removed, the brain retracted and the hypophysis exposed by transecting the infundibulum flush with the hypothalamus. The gland usually came out of the sella turcica in one piece, the posterior lobe being easily recoverable if it separated. The whole gland was blotted, weighed immediately to the nearest 0.5 mg, and dried to constant weight in an oven at 105°C in order to determine water content.

Results. From Table I it can be seen that the relative hypophysis weight in immature animals is greater (by 18.12%) than that of adults. The mean HW/BW of adult females was greater by 17.06% than that of adult males; there was no significant sex-linked difference, however, in puppies. Significant differences in relative pituitary weight were found between medium and large, and

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between small and large, but not between small and medium dogs. No sex difference was found within any single weight group of adult animals. Although the number of senile animals was small, their relative hypophysis size was distinctly (33.8%) and significantly less than that of adults.

No significant differences were found in water content of the hypophysis (range, 82.25% to 83.88%).

Discussion. The observations of relative hypophysis weights in pups and in adults, as far as sex difference is concerned, confirm those of other workers. Hypophysis weight seems subject to gonadal influence, being increased in the female by estrus(11), pregnancy(10), and the injection of chorionic gonadotrophin, theelin and theelol(12). The hypophysis of the male is not influenced, however, by the administration of testosterone. It would be expected, therefore, that the female hypophysis would be heavier.

Constancy of water content of the hypophysis in the several groups suggests that fluid shifts into and out of the gland are not important effects upon its relative weight. It would be of interest to study water content of the hypophysis in estrous females.

Summary. 1. It is confirmed in the dog, as has been shown for other species, that the relative weight of the hypophysis is greater in adult females than in adult males, and that this difference does not exist before maturity. 2. The hypophysis weight-body weight ratio of puppies is greater than that of adult dogs, and in adult dogs decreases significantly with increase in body size. 3. Relative hypophysis weight was smaller in senile than in adult animals. 4. No sex difference in hypophysis weight existed within any weight-group of adult animals. 5. No differences between groups could be detected with respect to water content of the hypophysis.

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Effect of Insulin Hypoglycemia on Eosinophiles and Lymphocytes of Psychotics.* (18048)

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The adrenal glucocorticoids, essentially the 11-17-oxysteroids, produce a fall in the circulating eosinophiles and lymphocytes(1,2). Eosinophile and lymphocyte counts have been utilized to evaluate the functional state of the adrenal cortex under various conditions of stress. Insulin hypoglycemia is one notable form of stress. An antagonism exists between insulin and adrenal glucocorticoids(3). Insulin hypoglycemia liberates epinephrine(4,5), which is an effective anterior pituitary-adrenal cortical activator(6,7).

The present report is a study of the effect of insulin hypoglycemia on the eosinophiles and lymphocytes of schizophrenic patients.

* The psychotic patients were made available through the cooperation of Dr. Jack R. Ewalt, Director, Galveston State Psychopathic Hospital.

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TABLE I.

No.	Age and sex	No. insulin injections	Insulin given I.M., units	Initial blood glucose (mg %)	Lowest blood glucose determined		Maximum % fall				Remarks
					hr*	mg %	hr*	Eos.	hr*	Lym.	
1	42 F	17	110	105	4	21	8	79	6	60	Drowsy
2	16 M	18	130	110	4	22	8	69	6	38	"
3	29 F	28	235	212	2	15	4	86	4	50	"
4	36 F	19	140	88	2	33	6	79	6	56	Coma
5	38 M	14	145	97	2	8	6	96	6	60	"
6	29 F	17	250	99	2	13	6	96	6	70	"
7	27 F	25	90	134	2	21	6	93	4	52	"
		27	90	122	1	65	2	37	2	16	Glucose†
8	38 F	12	340	120	7	51	4	27		‡	Glucose†
		17	250	—	2	23	6	75	6	64	Coma & EST
		22	250	122	4	18	6	87	6	47	"

* No. of hr after insulin administration.
† Glucose given concurrently with insulin.
‡ Lymphocytosis up to 39% at the end of 12 hr.

Eight schizophrenic patients were chosen at random for this study. They were hospitalized and had been receiving insulin treatment for varying periods of time. The essential clinical data are shown in Table I. Capillary blood samples were obtained from the finger. The blood glucose was estimated by the method of Folin and Malmros(8). Eosinophile counts were made with the Randolph direct method (9). Lymphocytes were calculated indirectly from the total white and differential counts of 400 or 500 cells. Control counts were obtained just before insulin was given. Additional counts were made every 2 hours for 12 hours.

The mechanism of the effect was further studied in Cases 7 and 8 by preventing hypoglycemia by oral and intravenously administered glucose. Four grams of glucose per unit of insulin were given within 4 hours of insulin administration and blood glucose was estimated at half hour intervals. Eosinophile and lymphocyte counts were also made when Case 8 received a combined insulin and electric shock treatment.

A uniform and significant decrease in both eosinophiles and lymphocytes was observed in all cases of insulin hypoglycemia. The eosinopenia amounted to 69 to 96% fall; and the lymphocytopenia 38 to 70% fall. The lowest blood glucose value obtained at 2-hour intervals occurred 2 to 4 hours after the administration of insulin. The maximal eosinopenia occurred 4 to 8 hours after insulin administration and 2 to 4 hours after the lowest blood glucose occurred. The maximal lymphocytopenia occurred 4 to 6 hours after insulin administration and 2 to 4 hours after the lowest blood glucose occurred.

The number of previous injections of insulin, the dosage of insulin and the age of the patient did not influence the magnitude of the decrease of either eosinophiles or lymphocytes. Nor did it seem that the clinical status was related to the extent of fall in this mixed group of improved and unimproved cases.

An added electric shock, which also causes eosinopenia(10) and lymphocytopenia(11) did not intensify the drop in Case 8 when electric shock was given during the period of insulin shock.

When glucose was given concurrently with insulin in Cases 7 and 8 and hypoglycemia prevented, the eosinopenia (37 and 27% fall respectively) was below the minimal 50% fall produced by 25 mg ACTH or 0.2-0.3 mg of epinephrine(12). There was also no signi-

8. Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, v83, 115.

9. Randolph, G. H., *J. Allergy*, 1948, v15, 89.

10. Altschule, M. D., Parhurst, B. H., and Tillotson, K. T., *J. Clin. Endocrinol.*, 1949, v9, 440.

11. Mikkelsen, W. R., and Hutchens, T. T., *Endocrinology*, 1948, v42, 394.

12. Thorn, G. W., and Forsham, P. H., *Recent Progress of Hormone Research*, v. 4, Academic Press, N.Y., 1949, 265.

ficant change in the lymphocytes (16% decrease in Case 8 and 39% increase in Case 9).

We have found no reports on eosinopenia following insulin hypoglycemia in either psychotics or normal individuals. Godlowski(13) demonstrated, however, a similar significant decrease in eosinophiles of eleven allergic patients with eosinophilia after the subcutaneous administration of 100 units of insulin. The period during which maximal eosinopenia occurred was 6 to 8 hours after the administration of insulin in his series. This is comparable to our results of 4 to 8 hours after intramuscular administration of insulin.

Lymphopenia following insulin hypoglycemia in psychotics has been observed by Katzenelbogen(14) and Parsons(15). The period during which maximal lymphocytopenia occurred in Parsons' series was 4 to 6 hours after intramuscular insulin administration, which is identical with our findings.

As mentioned above, insulin hypoglycemia causes an outpouring of epinephrine(3,4), which stimulates the production of ACTH by the anterior pituitary(6,7). It is probable therefore that the adrenocortical activation in insulin hypoglycemia depends on the liberation of epinephrine from the adrenal medulla. The quantitative and qualitative similarity of the falls of eosinophiles following insulin and epinephrine in allergic subjects, as observed by Godlowski(13), and the same similarity of the falls of lymphocytes following insulin and epinephrine in psychotics, as observed by Parsons(15), support this view. The delayed occurrence of eosinopenia and lymphocytopenia following insulin as compared with epinephrine and electric shock(13, 15) can be explained by the time interval required for the hypoglycemic effect of insulin and the subsequent output of epinephrine from the adrenal medulla to take place. When hypoglycemia was prevented by glucose (Cases 7 and 8) no significant eosinopenia and lymphocytopenia occurred. This also supports our belief that epinephrine is the cause of adrenocortical activation in insulin hypoglycemia.

Further studies, including the concurrent employment of adrenolytic drugs during insulin hypoglycemia, are in progress.

Summary. Insulin hypoglycemia causes in schizophrenics a significant eosinopenia and lymphocytopenia, which is considered to be indicative of adrenocortical stimulation. Insulin does not produce such changes if hypoglycemia is prevented.

13. Godlowski, Z. Z., *British Med. J.*, 1948, v1, 47.

14. Katzenelbogen, S., *Am. J. Psychiatry*, 1939, v95, 793.

15. Parsons, E. H., Gildea, E. F., Ronzon, E., and Herbeit, S. Z., *Am. J. Psychiatry*, 1949, v105, 573.

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Culture of Brown-Pearce Carcinoma in the Embryonated Egg. (18049)

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The Brown-Pearce carcinoma is commonly maintained by rabbit inoculation. Since the tumor may regress or die out during the summer months, and because of obvious economic and experimental advantages, we have investigated the feasibility of maintaining the growth in the developing egg. The present experiments show that the Brown-Pearce carcinoma may be cultured readily on the chorioallantoic membrane of the chick, and that it retains its capacity for growth and metastasis in the rabbit. Data are also presented on some conditions conducive to an optimal number of "takes" on the chorioallantoic membrane.

Materials and methods. Brown-Pearce tissue was obtained from female Dutch rabbits inoculated by the intra-uterine technic of

TABLE I.
Growth of Brown-Pearce Implants on Chorioallantois of the Chick Embryo.

Subculture	Total eggs	No. embryos surviving	No. surviving embryos with takes	% takes in surviving embryos
		Experiment A		
1	24	19	12	63.2
2	10	9	8	89.0
3	8	8	8	100.0
4	15	13	10	77.0
5	19	17	13	76.5
6	10	7	5	71.4
Totals	86	73	56	76.4
		Experiment B		
1	12	8	5	62.5
2	5	5	2	40.0
3	3	3	2	66.0
4	6	6	3	50.0
5	7	7	7	100.0
6	11	10	8	80.0
7	11	9	5	55.6
8	9	7	4	57.1
Totals	64	55	36	65.5
		Experiment C		
1	35	28	16	57.3
2	11	10	6	60.0
3	5	5	2	40.0
4	5	5	3	60.0
5	5	5	5	100.0
6	5	3	1	33.0
7	3	2	2	66.0
Totals	69	59	35	59.3
Grand totals	220	187	127	67.9
		*Experiment D**		
1	145	102	99	97.0

* Tumor tissue maintained *in vitro* in rabbit serum for 2 to 6 hr before implantation on the chorioallantois.

Dr. Elly M. Jacobsen.* Fourteen days after inoculation of the rabbits the metastasized tissue was removed aseptically and transplanted onto the chorioallantois of New Hampshire Red eggs which had been incubated for 8 days at 37.5°C. The chorioallantoic method described by Rugh(1) was used with the exception that the window in the shell was covered with 2 layers of sterile cellophane cut out of flat dialysis tubing and fastened to the shell with 1/4 inch wide strips of scotch tape. Pieces of tumor of approximately 1 mm cube were placed upon a large chorioallantoic vessel, and the sealed eggs were then maintained in the horizontal position without rotation for about 9 days. At this time the tumor mass, together with a portion of the adjacent membrane, was removed to a sterile saline-moistened paper towel in a watch glass. For subculture bits of tissue were immediately transferred to the chorioallantoic of new 8-day embryos. In several series the attached membranes were removed, excess fluid drained off with filter paper, and the wet weights obtained. Thirty-eight tumors were fixed in Bouin's fluid, sectioned at 8 μ and stained with Delafield's hematoxylin and eosin, and smears were made of blood from vessels serving the site of the tumor. The egg-cultured tumors were finally re-implanted into the rabbit *via* the anterior chamber of the eye in order to ascertain

* We are indebted to Dr. Jacobsen for providing us with tumor tissue and for generous aid and counsel.

1. Rugh, R., *Exp. Embryol.*, 1948, 441.

TABLE II.
Results Obtained with Single and Multiple Implants of Brown-Pearce Tissue on Chorioallantois of the Chick.

	No. surviving embryos	No. surviving embryos with takes*	% surviving embryos with takes
Single implants	70	41	58.5
Multiple implants	113	85	75.2

* The term "take" refers to the presence of one or more growths per egg.

possible alterations in the virulence of the growth.

Percentages of successful grafts. Table I summarizes the results of 3 experiments (A, B, C), each initiated with tumor tissue from a separate rabbit. The tissue was allowed to grow in the chick for 6-10 days (most commonly 9 days) and then grafted into a new group of eggs. The percentage of successful "takes" varies from 33% to 100% in various subcultures, with an average of 67.9% for embryos surviving to the 17th day of incubation. On the basis of the total number of eggs implanted 57.7% show successful grafts on the 17th day. It will be noted that there is apparently no trend toward increase or decrease of successful takes in successive subcultures.

In experiments A, B, C (Table I), the tumor tissue was placed upon saline-moistened paper towelling and portions were then immediately transplanted. In a second series of experiments, the excised tissue was separated into 1 mm cube fragments, incubated in rabbit serum for 2-6 hours at 37°C, and then transplanted as usual onto the chorioallantois. As indicated in Table I (Experiment D), the percentage of successful grafts was significantly increased, although we are unable to conclude that the superiority of this method is due to the serum rather than to the period of maintenance *in vitro,* incubation at 37°C, or to a combination of these factors.

The percentages of successful takes in eggs receiving only one tumor implant as compared with others receiving 2-4 implants per egg, are given in Table II. The apparent superiority of multiple grafts may possibly be due to injury of the tumor tissue during the course of subculture; however, observation suggests that implants frequently are displaced, whether by embryonic movements or other factors, from the original site of implantation. It may be suggested that the presence of several grafts makes it more likely that at least one of them will retain its position upon a large blood vessel.

Weight increase. In 3 series of grafts the growths were removed from the membrane after 8-9 days of culture, blotted with filter paper and weighed. On the basis of the tumors obtained from 37 eggs the wet weight of the tumor shows an average increase of 79 times over the weight of the original implant. Actually, the average growth rate must be far greater than is indicated by these values, since the active tumor mass originates from a small portion of the implanted tissue (Fig. 2). An indeterminate but certainly greater portion of the implant remains on the chorioallantois as a necrotic mass.

Re-implantation into rabbits. Tumors were removed from the chorioallantois at various subcultures and re-implanted into the anterior chamber of the rabbit eye. As shown in Table III, the egg-cultured tumors grew in every instance, irrespective of the age of the host, and there was definite metastasis in 8 of the 14 rabbits used. It is of interest to note that the adrenals were involved in 7 of the 8 animals showing metastases, whereas the mesenteries and serosa were affected in only one instance and the liver was entirely free of macroscopic growths.

Histology. The tumor *in situ* usually has the appearance of a pinkish mass adherent to the median surface of the chorioallantois. Smaller growths commonly show a thick whitish peripheral zone and a dark red central core; larger growths (approximately 10 x 10 x 10 mm) are almost entirely dark red, the light peripheral zone being extremely thin. The large vessels of the chorioallantois may

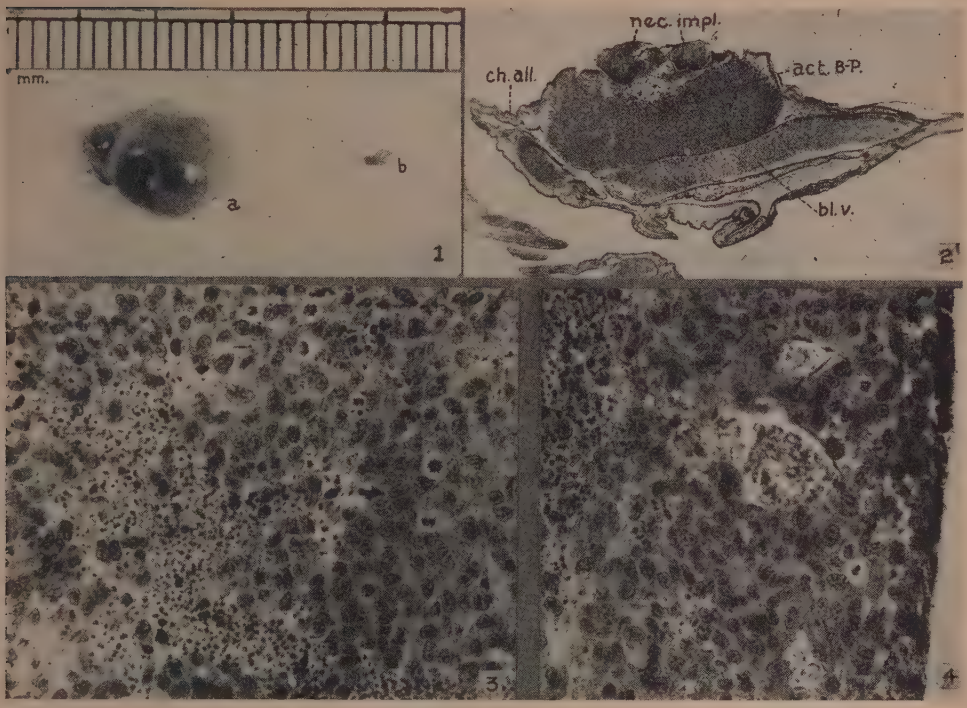

FIG. 1. Typical large tumor (a) cultured on the chorioallantois for comparison with piece of tumor used as implant (b).

FIG. 2. Section through a tumor of moderate size cultured on the chorioallantois. *nec. impl.*, necrotic implants. *act. B-P.*, active Brown-Pearce cells. *bl. v.*, chorioallantoic blood vessel. *ch. all.*, chorioallantois.

FIG. 3. Section showing the central region of a tumor grown on the chorioallantois. The large Brown-Pearce cells are seen in intimate contact with embryonic blood, without intervening endothelium.

FIG. 4. Section of a tumor showing a peripheral portion with endothelial cells visible and Brown-Pearce cells packed about the vessels.

be seen on the median aspect of the tumor (Fig. 2), indicating that the growth pushes the vessels before it toward the interior of the egg. The original implant is discernible as a dark brown or almost black necrotic mass imbedded in the membrane immediately above the actively growing tumor tissue (Fig. 2).

Thirty-eight tumors representing specimens taken from various subcultures through the 8th, present a quite uniform histological picture. The tumor cells proliferate locally in the mesodermal stroma of the membrane, deflecting the endoderm and large blood vessels (Fig. 2). In contrast with growths of similar size in the rabbit, even the larger tumors show little or no connective tissue stroma among the tumor cells. In further contrast with the larger tumor nodules in the rabbit, growths of corresponding size in the chick show no necrotic areas, the entire growth being composed of active Brown-Pearce cells as far as may be judged from histological characteristics (Fig. 3, 4). In the central region of the growth the tumor cells are intimately mingled with chick blood cells present in spaces which apparently lack an endothelial boundary (Fig. 3). In peripheral regions of the tumor, vessels lined with endothelium are closely packed with Brown-Pearce cells, but in no instance were tumor cells observed within the vessel (Fig. 4).

In view of the intimate contact of the

TABLE III.
Implantation of Brown-Pearce Tumor Grown on Chick Chorioallantois into the Anterior Chamber of the Normal Rabbit Eye.*

Animal No.	Rabbit age (mo.)	Source of tissue†		Growth in anterior chamber¶	Period of growth in anterior chamber (wk)	Metastases
		Exp.	Subcult.			
122‡	unknown	C	1	Yes	12	Possibly right adrenal
124	18	C	1	"	10	Nodules on serosa of gut, mesenteries, peritoneum and omentum
136	15	B	3	"	11	None
139	16	B	3	"	8	"
140§	12	B	1	"	6	Nodules on kidney, right and left adrenals
144	15	C	2	"	11	None
1	4.5	A	2	"	10	Right adrenal, right eye
2	4.5	A	2	"	10	" " " "
3	4.5	A	2	"	10	Right and left kidneys, right adrenal
4	4.5	A	2	"	10	None
5	5	B	5	"	9	Rt. kidney, rt. adrenal
6	5	B	5	"	9	None
7	5	B	5	"	9	Right adrenal
8	5.4	C	4	"	9	Left "

* Unless otherwise indicated, implantation was made into the left eye.
† Tumor tissue taken from the experiments and subcultures indicated in Table I.
‡ Implantation into right eye.
§ " " both eyes.
¶ Growths in all instances completely filled the anterior chamber; in many the entire eye was replaced.

tumor cells with the embryo's blood, especially in the central region, it is surprising that no metastases could be found, although most of the chicks were autopsied with this purpose in mind. Furthermore, histological examination of the embryonic liver, spleen, gall bladder, mesonephros, heart, and brain in a number of specimens gave no evidence of metastases nor of any pathological change. Smears made of blood from vessels immediately supplying and draining the tumor site showed no Brown-Pearce cells in any instance. The only observed effects of the tumor on the chick are limited to the localized region of the chorioallantois overlying or surrounding the growth. Here the membrane is translucent and slightly wrinkled, and may show ectodermal proliferation and apparent keratinization as already described by Campbell (2). The chick proper shows no apparent effect of the tumor and a number of specimens allowed to hatch, observed for several weeks, and then autopsied were quite normal.

The failure of the tumor to metastasize in the chick may depend upon an antagonistic factor present in the chorioallantois and is worthy of further study. The anti-metastatic effect does not persist after removal of the tumor from the chick, since metastasis occurs upon re-implantation into the rabbit. Lee *et al.*(3) obtained metastases of mouse tumor by injecting the tumor into the allantoic vein, which suggests that the vascular wall possibly acts as a barrier. However, this seems a highly improbable explanation insofar as the Brown-Pearce tumor is concerned, since the tumor cells appear to be in direct contact with the embryonic blood in the central region of the growth.

Summary. 1. The Brown-Pearce carcinoma of rabbits shows definite growth on the chorioallantois in 58% of embryos implanted on the 8th day of incubation, and in 68% of embryos surviving until the 17th day. The wet weight of the tumor increases 79 fold

2. Campbell, J. G., *Brit. J. Cancer*, 1949, v3, 72.

3. Lee, H. F., Bender, D. H., and Friedgood, C. E., *Science*, 1948, v107, 374.

during the 9-day culture period. 2. The average percentage of successful grafts is increased by implantation two or more pieces of tumor within a single egg, and by incubating the excised tumor for several hours in rabbit serum before implantation onto the chorioallantois. 3. The tumor does not metastasize from the chorioallantois in spite of apparent direct contact between the embryonic blood and the tumor cells.

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Use of Antipyrine in Measurement of Total Body Water in Animals.* (18050)

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A method for determining total body water in man by means of antipyrine has recently been described(1). In a series of tests this drug fulfilled the characteristics a substance should possess for the measurement of total body water in man: It was not toxic in the amounts necessary for the determination, it was distributed evenly and rapidly throughout the body water, it was degraded and excreted at a regular rate, and the analytical procedure for its determination was accurate and simple.

Most methods for measuring total body water are based on the volume of distribution of a foreign substance after intravenous administration. These procedures are in the main unsatisfactory because of unequal distribution in the tissue water of the body. In contrast, determining total body water with deuterium oxide or tritium is more satisfactory as these isotopes distribute evenly, but the analytical technics are difficult and the cost high(2,3,4).

Antipyrine is rapidly transformed in the dog and it was thought that this characteristic would preclude its use for the measurement of total body water. The following studies were carried out to ascertain whether or not antipyrine is in fact satisfactory for this purpose.

Methods. Measurement of antipyrine in tissues and plasma has been described‡(5). Following intravenous injection, the distribution of antipyrine was determined in representative tissues of one rabbit at 50 minutes following injection and 2 dogs at 1½ hours and 2¾ hours respectively. The animals were killed by intravenous air injection and the tissues were analyzed immediately.

The following technic was employed to measure total body water: control sample of blood was withdrawn to determine the plasma blank value for correcting subsequent samples; 75 mg of antipyrine per kilo body weight in the form of a 5% solution in distilled water was injected intravenously from a burette or calibrated syringe; blood samples were withdrawn and heparinized at 2, 3, and 4 hours after injection; plasma and cells were

* The technical assistance of James J. Dooley and Mrs. Mary M. McLaughlin is gratefully acknowledged.

† Present address: Bellevue Hospital, Third New York University Medical Division, New York, New York.

1. Soberman, R., Brodie, B., Levy, B., Axelrod, J., Hollander, V., and Steele, J. M., *J. Biol. Chem.*, 1949, v179, 31.

2. Pace, N., Kline, L., Schachman, H. K., and Harfenist, M., *J. Biol. Chem.*, 1947, v168, 459.

3. Hevesy, G., and Hofer, E., *Nature*, 1934, v134, 879.

4. Moore, F. D., *Science*, 1946, v104, 157.

‡ The method is based on the addition of sodium nitrite to a plasma filtrate or tissue extract with the resulting formation of a 4-nitroso antipyrine which can be read in the spectrophotometer at 350 mu. Appropriate dilutions of the plasma are made so that readings fall in the significant colorimeter range.

5. Brodie, B., Axelrod, J., Soberman, R., and Levy, B., *J. Biol. Chem.*, 1949, v179, 25.

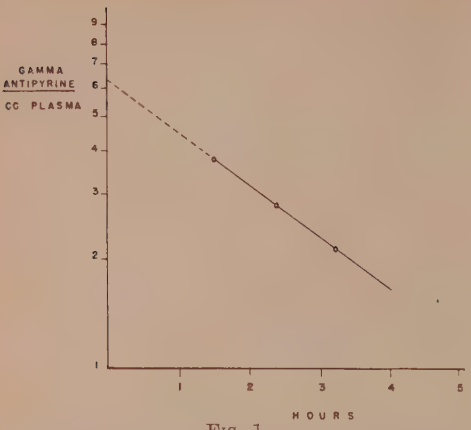

FIG. 1.

Plasma levels of antipyrine. The curve for the plasma levels is extrapolated to zero time to correct for the metabolism of the drug during the time required for uniform distribution.

separated after centrifugation and the plasma stored in stoppered tubes for subsequent antipyrine analysis.

The plasma concentration at zero time (the concentration at the time of injection, if uniform distribution had been instantaneous and if none of the substance had been metabolized) was calculated by plotting the plasma levels on semilogarithmic paper and extrapolating the straight portion of the time-concentration curve (2nd, 3rd and 4th hours) back to the time of injection (Fig. 1).

The plasma water level of antipyrine was calculated by dividing the plasma level of antipyrine by the water content of the plasma (.93 average).

The calculation for total body water was made as follows:

$$\text{Body water (liters)} = \frac{\text{amount of drug injected (mg)}}{\text{plasma water level (mg per liter)}}$$

To obtain a comparison of antipyrine and desiccation methods, 5 monkeys, 4 dogs, and 4 rabbits were used. After total body water had been determined with antipyrine the animals were weighed and sacrificed. The entire animal was ground, and the final mass then spread over pans and placed in an oven at a temperature of 95°C for a period of 4 to 7 days until constant weight was achieved.

Results. The ratio of tissue water antipyrine concentration divided by plasma water antipyrine concentration was found to be close to unity in all tissues, with three exceptions. These were in the liver and kidney where degradation and excretion were presumably taking place, and in the lung. The antipyrine concentration in tissues appeared to be related to their water content (Table I).

Values for total body water in 4 dogs, 5 monkeys, and 4 rabbits obtained by antipyrine and by desiccation did not differ significantly (Table II). The total body water in terms of per cent body weight ranged from 62.8% to 77.8% with an average of 70.9%. The average difference was 54.5 cc, with values ranging from 10 to 122 cc. The coefficient of correlation was 0.93.

After plotting the plasma concentration as a semilogarithmic function of time, it was observed that in all species tested, a linear relationship was obtained within two hours after the injection of antipyrine. The smaller the animal, the earlier even distribution was achieved; *e.g.* in the small rabbits, even distribution was achieved after 50 minutes.

In an effort to ascertain whether the rate of transformation of antipyrine was regular when even distribution had been achieved (for extrapolation purposes), 75 mg of antipyrine per kilo of body weight was administered intravenously to 20 dogs, 5 monkeys, and 4 rabbits. Blood samples were taken at intervals following complete distribution and

TABLE I.
Distribution of Antipyrine in Water of Animal Tissues.

Tissue	Ratio: tissue water antipyrine / plasma water antipyrine		
	Dog 1	Dog 2	Rabbit
Plasma	1.00	1.00	1.00
Heart	1.07	1.04	.96
Muscle	1.05	0.94	.99
Muscle	1.05	1.00	
Spleen	1.03		
Kidney	1.12	1.12	.97
Liver	1.04	1.10	1.08
Lung	0.83	0.86	1.01
Brain	0.99		
Cerebrospinal fluid	0.88	0.99	
Average	1.06	1.01	1.00

TABLE II.
Comparison of Total Body Water in Animals Determined by Antipyrine and Desiccation.

Animal	Weight (g)	Total water: Antipyrine (cc)	Total water: Antipyrine (% body wt)	Total water: Desiccation (cc)	Total water: Desiccation (% body wt)	Difference in (%)
1. Monkey	3637	2290	62.8	2412	66.4	—3.6
2. "	3321	2360	71.0	2317	69.8	+1.2
3. "	2854	1980	69.4	2046	71.6	—2.2
4. "	3496	2350	67.2	2443	69.9	—2.7
5. "	3099	2230	72.1	2146	69.4	+2.7
6. Dog	875	662	75.7	623	71.3	+4.4
7. "	1303	832	63.9	886	68.1	—4.2
8. "	3279	2272	69.5	2373	72.5	—3.0
9. "	2891	2139	73.9	2097	72.5	+1.4
10. Rabbit	1782	1330	74.6	1350	75.8	—1.2
11. "	1846	1270	68.8	1285	69.6	—0.8
12. "	660	501	75.8	481	72.9	+2.9
13. "	1172	912	77.8	902	77.0	+0.8

the plasma analyzed for antipyrine. Results indicated that the average fall in blood level due to degradation and excretion was 30% per hour and varied from 20 to 50%. However, in a given animal the rate was constant over periods up to 7 hours. Thus, the transformation of antipyrine as represented by the falling plasma level could be corrected by extrapolation of the semilogarithmic curve of the plasma concentration to zero time.

Antipyrine was found to be non-toxic in 30 dogs, 10 rabbits and 8 monkeys given 75 mg of antipyrine per kilo body weight intravenously, with the exceptions that 5 dogs exhibited excessive salivation and one dog vomited immediately following a rapid injection. It was thought that the vomiting could have been avoided by a slower rate of injection.

Discussion. It was recently shown that the total body water content in adult humans as determined by antipyrine and deuterium ranged from 40 to 60% of body weight(1). The values of total body water showed considerable variability and suggested a clear inverse relationship between the amounts of total body water and total body fat. Higher values for total body water of 65 to 70% body weight were obtained with antipyrine on trained lean individuals(6). These values were confirmed by calculating total water from specific gravity determinations done on these same subjects, assuming that fat free tissue is 71.8% water.

The average value for total body water in the animals of this series was 70.9% body weight, ranging from 62.8 to 77.8% body weight. It should be emphasized that these animals were young and lean, purposely selected in order to facilitate desiccation. In contrast, the total body water determined in ten adult dogs in connection with other experiments ranged from 53.2 to 65.8% of the body weight with a mean of 58.5%. Thus, the values of total water above 71.8% body weight indicated that the assumption that 71.8% of the fat free tissue is water is invalid. The discrepancy may be due to differences in bone density and structure not taken into account in the calculation of total body water from specific gravity determinations.

Although the rate of transformation of antipyrine in animals is relatively rapid as compared to humans, this factor does not interfere with determination of total body water with antipyrine since it has been shown that the rate of transformation is constant over the period of time necessary for the determination.

Summary. Antipyrine is distributed evenly in the various tissues of animals in close proportion to their water content. The values obtained for total body water by antipyrine and by desiccation agreed well and gave a correlation coefficient of 0.93. The rate of

6. Osserman, E. S., Pitts, G. C., Welham, W., and Behnke, A. R., *J. Applied Phys.*, in press.

transformation and excretion of antipyrine as determined by plasma analysis was constant over a period of time sufficient for analysis.

Ease of analysis and lack of toxicity make antipyrine a suitable substance for use in measurement of total body water in animals.

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Infection and Immunity in Offspring of Mice Inoculated during Gestation with Murine Poliomyelitis Virus. (18051)

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A study of 100 pregnant mice, inoculated at various intervals during gestation with Col. SK murine poliomyelitis virus, has shown that there is a progressive increase in susceptibility to infection which begins with the fourth day of pregnancy and reaches a maximum during the last 4 days before parturition (1). The present paper deals with observations on the young from the inoculated mothers, the data being concerned with the infection of the fetus and the immunity of surviving offspring.

Materials and Methods. Among 100 gestating mice orally inoculated with Col.SK virus pregnancy terminated in 5 different ways (Table I). In addition to surviving young from these infected mothers, 6 litters whose mothers had been infected soon after parturition, another 6 litters produced by breeding mice with known immunity, and 4 normal litters contributed to the study of immunity in young mice.

Infection in the young. Fetal tissues, and tissues from offspring of mothers which had succumbed to infection after parturition were tested for recovery of virus by intracerebral inoculation of young mice.

Immunity of offspring. Litters were examined for their immunity at various intervals between weaning and 44 days of age by intranasal inoculation of Col.SK virus diluted 10^{-2}. Older littermates were tested by intraperitoneal inoculation of virus diluted 0.5 x 10^{-2}, a challenge which served also in investigating the development of maternal immunity. Surviving offspring of paralyzed mothers were placed with litters of other lactating mice, some of which were normal, whereas others were surviving experimental animals. When it became apparent that immunity in these fostered infants corresponded with the immunity of the fostering mother, the influence of suckling with immune mice was further investigated by means of interchanging normal baby mice and the young of immune mice during the 21-day period of nursing, and subsequently testing the fostered mice for their ability to resist infection.

In vitro test of milk for its capacity to neutralize virus. Milk collected(2) from 2 immune mothers was pooled and examined at various dilutions for its capacity to neutralize Col.SK virus. Pooled milk from 2 normal lactating mice served as control. Equal mixtures of milk and suspensions of virus were incubated at 37°C for 1 hour, placed overnight in the refrigerator, and then inoculated intraperitoneally into young mice.

Results. Outcome of pregnancy. (Table I). Eighteen viable litters, born to 100 female mice orally inoculated with Col.SK virus during gestation, were apparently normal at birth. One half of the litters were reared by their own mothers which had resisted infection. The remaining 9 litters came from mothers which became paralyzed after parturition; 4 of these litters were successfully reared by foster mothers. The rate of abortion was high among mice infected

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TABLE I.
Outcome of Pregnancy in Mice Orally Inoculated with Col. SK Virus During Gestation.

Time of inoculation of pregnant mice. Gestational days	Litters of 25 surviving mice			Litters of 75 paralyzed mice				
	Living	Devoured at term	Aborted	Living	Stillborn or died shortly	Devoured at term	*In utero* at death of mother*	Aborted
1 to 5	4	0	6	0	0	0	7	4
6 to 10	1	0	7	0	0	0	18	3
11 to 15	2	2	1	3	7	3	12	3
16 to 19	2	0	0	6	5	2	2	0
Totals	9 (36%)	2 (8%)	14 (56%)	9 (12%)	12 (16%)	5 (7%)	39 (52%)	10 (13%)

* Of the litters found *in utero*, 26 gave evidence that fetal death had preceded maternal death; 21 of these came from mice inoculated between the 1st and 10th days of gestation, inclusive.

TABLE II.
Recovery of Virus from Tissues of Young.

Time of onset of maternal symptoms	Condition of litter	Recovery of virus from tissues of fetuses or young No. of tests	
		Positive	Negative
Before parturition	Died *in utero*	13	0
At parturition	Stillborn	3	1
	Sacrificed at birth	0	1
	Died at 2 days of age*	0	1
Postpartum: 24 hrs	Sacrificed at birth	0	1
	Died at 3 days of age*	1	0
48 "	Sacrificed at death of mother	0	2
	Died at 4 days of age*	0	1
5 days	Sacrificed at death of mother	0	1

* Fostered 2 days by normal foster mother.

during the first 10 days of gestation, varying from 72% in the case of resistant animals to 88% for those that died of the disease. With inoculation carried out after mid-term, however, abortion was comparatively rare.

Infection in the young. (Table II). Fetuses recovered at autopsy from paralyzed mice were found invariably to have been infected *in utero*, whereas young mice born during the maternal incubation period of the disease as a rule escaped infection. This indicates that intrauterine infection occurs but late in the course of the maternal disease.

Immunity in the young. Resistance of young mice to intranasal infection with Col. SK virus correlated well with the development of immunity in the mother (Table III). The susceptibility of littermates examined after the age of 4 months illustrates the transient nature of the observed immunity in the young. This immunity was shown to be directly related with the immunity of the nursing mother which suckled the babies, rather than with the immunity of the natural mother (Table IV). The time required to produce immunity in baby mice through suckling with immune mothers was not determined. It was observed, however, that 5 litters which had been placed with immune mothers, not on the day of birth, but at various times between the third and eleventh days of age, were fully protected from infection. Thus, a nursing period of 10 days was sufficient to provide complete protection, and a delay of 11 days between birth and fostering with an immune mother did not preclude the development of immunity in the fostered young.

In vitro capacity of milk to neutralize virus. (Table V). Milk obtained from normal lactating mice contained no substances capable of neutralizing Col.SK virus, while

TABLE III.
Immunity of Surviving Mothers and Their Offspring.

Mothers			Offspring tested for immunity at various ages Results of inoculation with virus at:		
Time of oral infection with virus	No.	Immunity as shown by intraperitoneal test	23 to 44 days of age	66 to 68 days of age	over 119 days of age
Inoculated during pregnancy	5	Positive	0/26		4/4
	2	Negative	10/10		2/2
	1	Not tested	4/4		3/4
Inoculated soon after parturition	6	Positive	3/19*	5/7	
Inoculated during first pregnancy and rebred	3	Positive	1/12		5/6

Denominator: No. of mice inoculated; numerator: No. of mice died.
* Three mice which died comprised 1 litter.

TABLE IV.
Immunity of Young Mice Following Fostering with Normal or Immune Mothers.
(Tested between 23 and 36 days of age).

Immunity of natural mothers	Immunity of offspring. Results of intranasal inoculation with virus after suckling with:	
	Normal foster mother	Immune foster mother
Negative	7/7 (members of 3 litters)	0/16 (members of 7 litters)
Positive	5/5 (members of 2 litters)	0/6 (members of 2 litters)

Denominator: No. of mice inoculated; numerator: No. of mice died.

TABLE V.
Capacity of Milk to Neutralize Col. SK Virus *in Vitro*.

Milk		Virus dilutions	
Source	Dilution	10^{-2}	10^{-4}
Immune mice	Undiluted	S, S, S	S, S, S
	1:5	7, S, S	S, S, S
	1:10	3, 3, 4	S, S, S
	1:50	3, 3, 4	6, 8, S
	1:100	3, 4, 4	6, 7, S
Normal mice	Undiluted	2, 3, 3, 4	3, 4, 4, 5
	1:10	2, 3, 3, 3	4, 5, 5, 6

Numbers indicate incubation periods (in days) of paralyzed test mice. "S" indicates surviving test animal.

milk collected from immune mothers effectively neutralized the virus. In the absence of any other explanation, it is believed that the neutralizing principle in the milk of immune mothers was the same antibody which is usually present in the serum.

Discussion. From the data presented it would appear that murine poliomyelitis, occurring during gestation, frequently interferes with the successful outcome of pregnancy. This conclusion is based upon two observations: the low rate of productivity among susceptible animals infected during the last few days of pregnancy, and the high rate of abortion among animals inoculated before mid-term. A similarly high rate of abortion

has been observed by Byrd(3) in pregnant mice infected intracerebrally with the Lansing strain of murine poliomyelitis virus. The constant presence of virus in the fetuses of paralyzed mothers suggests the primary nature of the risk to the young *in utero* and militates against the presence of a murine placental barrier to the virus. On the other hand, the fact that living, normal litters may be born while mothers are in the incubation period of the disease indicates that embryos are well protected from infection during the initial phases of the disease process. Only one of such litters proved to be infected, and, judging from the time of onset of paralysis, probably had contracted the disease during or soon after birth rather than *in utero*.

As far as our data go, there is a close correspondence between resistance of the young and immunity of the mouse which suckles them. Similar results were obtained by Orskov and Andersen(4) who found that litters were passively protected against oral infection with Theiler-FA virus or with Col. SK virus when mothers had previously been vaccinated with the corresponding virus. No cross immunity was observed in these tests. In work with MM virus, Curley and Gordon (5) and Anderson and Bolin(6) have demonstrated the passive nature of immunity in the young and its brief duration, and have shown that such resistance is associated with the immunity of the mouse which suckles the young(7). Transfer of antibody by way of ingested milk was postulated as the mechanism responsible for the passive protection of young mice, although the transplacental route was also considered. The latter mechanism, however, granted a protection of much shorter duration(7). While, in the mouse, passive transfer of immune bodies of various types undoubtedly does occur *in utero*, the most important route in this species is thought to be by way of the milk(8,9). In view of the constant agreement obtained by *in vivo* experiments, the *in vitro* demonstration of virus neutralizing substances in the milk of immune mice is not surprising. Furthermore, the fact that milk obtained from normal mice exhibited no neutralizing capacity establishes the specific nature of the phenomenon.

Summary. 1. From 100 mice, orally infected with Col.SK virus at various times during the period of gestation, only 18 viable litters were obtained. These litters were equally divided among 25 surviving animals and 75 animals which succumbed to the disease.

2. The rate of abortion for mice inoculated during the first half of pregnancy, regardless of their capacity to resist infection, was high, being 72% for surviving animals and 88% for those which died, since two-thirds of the litters found *in utero* at the time of maternal death gave evidence of having predeceased the mother.

3. Fetuses obtained at autopsy consistently contained demonstrable amounts of virus.

4. Living young, when delivered at the time of onset of maternal symptoms, had apparently escaped infection, while a majority of stillborn young yielded virus.

5. Surviving offspring of susceptible mothers, born during the maternal incubation period, were apparently normal and usually escaped infection.

6. Litters of immune mothers, with one exception, were fully protected against intranasal infection between weaning and the age of 44 days. When tested between 66 and 68 days of age only 2 of 7 young were resistant, while after 119 days there was no evidence of immunity.

7. By means of experiments in which the offspring of immune and normal mice were interchanged during the period of suckling, immunity in the young was shown to be re-

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lated to the observed immunity in the nursing mother.

8. Milk collected from normal mice contained no antiviral substances while milk from immune mice was capable of neutralizing the virus *in vitro*.

9. The data indicate that the protection of young mice is passive in nature and probably brought about by the ingestion of antiviral substances (antibodies?) in the milk of immune mothers. There is no evidence in this work to suggest that the offspring of infected mothers may acquire a state of active immunity through inapparent infection.

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Effect of Purified Hyaluronidase on Growth of Sarcoma 37 in the Mouse. (18052)

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Several studies of the effect of hyaluronidase on the growth and invasiveness of experimental tumors have been reported with contradictory results ranging from enhancement (1,2) to inhibition (3,4) and no effect (5,6,7). In all these studies crude preparations and undetermined doses of enzyme were administered. It seemed desirable, therefore, to carry out further studies with massive doses of a highly purified hyaluronidase of the quality used as a therapeutic and diagnostic agent.*

Materials and methods. One hundred and sixty-three male Swiss mice weighing between 17 and 20 g were implanted subcutaneously near the axilla with fragments of sarcoma 37 measuring approximately 2 sq mm.† The animals were injected daily either intravenously or subcutaneously with physiological salt solution (PSS), hyaluronidase dissolved in PSS, or heat inactivated hyaluronidase in PSS, as outlined in Table I. In Group VII the tumor implant consisted of a standardized macerated suspension of a 7 days old tumor mixed with 20 mg hyaluronidase. The subcutaneous injections were administered apart from the immediate site of tumor implantation. The hyaluronidase* was prepared from bull testes and assayed 6300 turbidity reducing units per mg of nitrogen. Inactivated hyaluronidase was prepared by heating the enzyme at 60°C for 2 to 6 hours. All injections were begun either on the day of implantation or on the seventh day following implantation. Thirteen to 16 days after implantation the mice were killed and the tumors dissected out and wet weighed. Sections of the following organs and tissues were examined for metastases by Dr. W. E. Ehrich of the Philadelphia General Hospital: liver, lungs, thymus gland, kidneys, salivary and mesenteric lymph nodes, adrenal glands, thyroid gland, salivary glands, pituitary gland, and bone marrow.

Results. Intravenous administration. In Group I in the control series of mice, which received PSS only, the tumors grew to a size which was significantly larger than those in mice which had received 10 mg hyaluronidase daily from the day of implantation. In Group

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* Wydase, Wyeth.

† A transplant of mouse sarcoma 37 was kindly supplied by Dr. H. J. Creech of the Lankenau Hospital Research Institute.

TABLE I.
Effect of Hyaluronidase on the Growth and Invasibility of Mouse Sarcoma 37.

Group	No. of mice	Material injected	Amt inj. daily (mg)	Period of inj. (days)	Total dose inj. (mg)	Route of inj.	Tumor wt* (g)	Lymph node metastasis
I	4	Hyaluronidase	10	10	100	Intrav.	.21	No exam.
	4	"	10	6‡	60	"	.32	" "
	4	PSS†	—	10	—	"	1.12	" "
II	10	Hyaluronidase	30	11	330	"	.77	1
	9	"	30	7‡	210	"	.42	3
	8	PSS	—	11	—	"	.70	1
III	11	Hyaluronidase	20	11	220	Subcut.	.089	0
	13	PSS	—	11	—	"	.34	0
IV	8	Hyaluronidase	10	11	110	"	.25	1
	8	Heat inactiv. hyaluronidase	10	11	110	"	.44	0
	8	PSS	—	11	—	"	.44	1
V	10	Hyaluronidase	2	9	18	"	.86	5
	10	Heat inactiv. hyaluronidase	2	9	18	"	.40	5
	9	PSS	—	9	—	"	.45	1
VI	7	Hyaluronidase	2	9	18	"	.27	1
	6	Heat inactiv. hyaluronidase	2	9	18	"	.32	1
	8	PSS	—	9	—	"	.67	0
VII	12	Hyaluronidase	2	10	40§	"	.56	0
	14	PSS	—	10	—	"	.42	0

* Log geometric mean.
† Physiological salt solution.
‡ Initial injection 7 days following tumor implantation.
§ Includes 20 mg hyaluronidase injected with tumor cell suspension.

II, where the mice received more hyaluronidase than in any one of the other groups (30 mg daily), two experiments carried out concurrently (except that the initial injection of hyaluronidase in one experiment was 7 days following the tumor implantation rather than on the same day) indicate that there was no striking effect on the growth or invasibility of sarcoma 37 (Table I).

Subcutaneous administration. A striking inhibitory effect on the growth of sarcoma 37 was shown by mice which received 20 mg hyaluronidase daily from the day of implantation (Group III). Where one-half this amount of enzyme was injected (Group IV) the tumors were likewise smaller than those in the PSS or inactivated hyaluronidase series. Of 2 groups of mice receiving 2 mg hyaluronidase daily (Groups V and VI), enhancement of tumor growth occurred in only one group. Mice injected with tumor cell suspension mixed with 20 mg hyaluronidase and then treated daily with 2 mg hyaluronidase (Group VII) did not show an increase in size of tumors as compared with the saline controls.

Microscopic examination of the liver, lungs, thymus gland, kidneys, thyroid gland, adrenal glands, salivary glands, pituitary gland, and bone marrow did not reveal metastasis.

Discussion. Because of the spreading action of hyaluronidase it might be assumed that the enzyme enhances the growth of tumors and encourages metastasis. However, a tumor probably requires the ground substance of connective tissue for growth. The intercellular ground substance cement is rich in hyaluronic acid, a mucopolysaccharide which is depolymerized by hyaluronidase. This enzyme, therefore, might conceivably even interfere with tumor growth, at least

until a sufficient antihyaluronidase titer had developed to prevent the enzyme from reacting with the mucopolysaccharide.

Although the data obtained are not altogether consistent, hyaluronidase did not appear to enhance the growth or invasibility of the tumors. The average weight of all the tumors of mice which received hyaluronidase is 18% lower than the average for the tumors in the PSS control series. Neither the concentration of enzyme nor the route of administration was a determining factor in the rate of tumor growth.

Summary and conclusions. 1. One hundred and sixty-three male Swiss mice weighing between 17 and 20 g were implanted subcutaneously with fragments of sarcoma 37 and then injected subcutaneously or intravenously beginning on the day of implantation or 7 days thereafter with hyaluronidase, heat inactivated hyaluronidase, or physiological salt solution.

2. The results on the effect of hyaluronidase administered subcutaneously or intravenously were somewhat variable, from which it is concluded that there was no effect on the growth or invasibility of implanted sarcoma 37. Neither was there metastasis to distant organs.

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Growth Retardation of Lymphosarcoma Implants in Pyridoxine-Deficient Rats by Testosterone and Cortisone. (18053)

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Previously it was shown that pyridoxine is more important than other dietary essentials for the maintenance of normal(1) and of neoplastic(2) lymphoid tissue. The administration of "glucocorticoids" as well as of androgenic steroids, like pyridoxine deprivation, produces a striking loss of normal lymphoid tissue(3). An influence of adrenal cortical secretion upon neoplastic lymphocytes was first demonstrated by Murphy and Sturm(4). Actual regression of lymphosarcoma transplants in mice, though only temporary, was observed by Heilman and Kendall (5) following the administration of cortisone. Androgens have so far not been reported to affect neoplastic lymphoid tissue and it has been concluded by Biesele and Gasic(6) that testosterone propionate does not diminish the chromosome size of neoplastic lymphocytes as it does that of normal lymphocytes. In the present experiments, the effect of cortisone was studied in lymphosarcoma-bearing rats, rendered deficient in vitamin B_6 and was compared to that of methyl testosterone.

Fifty male rats of the Sprague-Dawley strain of about 200 g body weight were divided into 7 groups as indicated in Table I. They were fed a diet composed of Casein Labco, 30%; Dextrose, 56%; Crisco, 10%; U.S.P. Salt Mixture No. 1, 4%; Thiamine, .001%; Riboflavin, .002%; Pyridoxine, .001%; Ca Pantothenate, .01%; Nicotinamide, .01%; Choline, 0.1%; Inositol, .05% except as follows: for Groups V, VI and VII, Pyridoxine HCl was omitted from the ration as indicated in the table. Cortisone acetate and methyl testosterone, suspended in saline, were ad-

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TABLE I.
Effect of Cortisone and of Methyl Testosterone Upon Growth of Lymphosarcoma Implants in Pyridoxine Deficient Rats.

Exp.	Group	Diet	Injection	Tumor wt, g (estimated) 1 wk	2 wk	3 wk	Days avg survival	No. of rats (male)
A	I	Complete	None	8	53	71	—*	6
	II	"	Cortisone (2 mg)	6	42	55	—	6
	III	"	Meth. test. (2 mg)	7	23	46	—	6
B	IV	Complete	None	5	31	61	20	8
	V	B_6 defic.†	"	4	18	25	23	8
	VI	" "	Cortisone (2 mg)	3	7	13	18	8
	VII	" "	Meth. test. (2 mg)	3	8	15	26	8

* Not determined.
† B_6 deficient diet + 2 mg of desoxypyridoxine daily.

ministered daily by subcutaneous injection. Two mg of desoxypyridoxine was fed daily by stomach tube to the animals of Groups V, VI and VII. The rats in Groups I to VII were inoculated under sterile precautions with fragments of the Murphy rat lymphosarcoma*. The implants were placed under the skin of the lower back. Twice weekly the animals were weighed and measurements taken of 3 cross diameters of the tumor implants. The approximate weight of the tumors was derived from a standard curve obtained from a control series in which the calculated volume was compared with the actual weight of dissected tumors. The dietary regimen and the injections in experiments A and B were begun the day after inoculation.

The findings summarized in Table I show that daily injections of cortisone and of methyl testosterone cause moderate but definite retardation of growth of the lymphosarcoma implants (Exp. A, Groups I, II and III). Comparing the 4 groups in experiment B, it is seen that "acute" pyridoxine deficiency (Group V) causes a depression of lymphosarcoma growth which was greater than that produced by either one of the two steroids (Groups II and III). However, the administration of cortisone or of methyl testosterone to animals deprived of vitamin B_6 (Groups VI and VII) resulted in marked suppression of the growth of the tumor implants. As in the animals on the complete diet, this action of cortisone was no greater than that of methyl testosterone.

In mice inoculated with lymphosarcoma (6C3H-ED) we have observed, in accordance with previous reports(5), that treatment with cortisone produced actual regression of this neoplasm. If cortisone treatment was combined with administration of the antivitamin, desoxypyridoxine, very rapid disappearance of the lymphosarcoma implants occurred; well established tumors disappeared within 2 days. This rapid regression, however, was invariably followed by death of the mice. From the present observations on a rat lymphosarcoma, reduction of survival time (Group VI) is less drastic but appeared significant from the fact that 3 rats in this group died much sooner than any animal in the other groups. Experiments, to be reported in detail elsewhere, have shown that early death in pyridoxine-deficient, cortisone-treated animals occurs similarly in non-tumor bearing rats and that the prolongation of life of rats treated with testosterone was much more striking in pyridoxine deficiency uncomplicated by neoplastic disease.

The augmentation of dietary protein is known to increase pyridoxine requirements and it appears that enhanced utilization of amino acids is associated with a greater requirement for vitamin B_6(7). The action of

* Tumor obtained through the courtesy of Dr. J. B. Murphy.

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"glucocorticoids" presumably causes an increased utilization of amino acids which is associated either with inhibition of protein anabolism or with enhancement of protein catabolism. Consequently, an excessive demand for pyridoxine may be expected to result from the administration of 11-oxygenated adrenal steroids or of substances with similar activity. Conversely, androgenic steroids having a metabolic effect opposite to that of "glucocorticoids" may exert a sparing action upon vitamin B_6. Evidence supporting these possibilities has been obtained from the present experiments. Furthermore, both cortisone and testosterone were found to accentuate the retardation of lymphosarcoma growth associated with lack of pyridoxine. This effect in the case of testosterone was accompanied by an alleviation of the nutritional disease.

Summary. Marked retardation of growth of lymphosarcoma implants was observed in pyridoxine-deficient rats injected with 2 mg daily of cortisone or of methyl testosterone. Survival of pyridoxine-deficient, tumor-bearing rats was prolonged by testosterone and shortened by cortisone.

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Studies on the Early Phase of Induced Fibrosarcoma in the Hamster. (18054)

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We have been able to produce fibrosarcomas in the golden hamster by injection of 20-methylcholanthrene in tricaprylin or olive oil. Similar sarcomas have been produced in the same species by the injection of 9,10-dimethyl-1,2 benzanthracene(1). The incidence of tumors was high, and the true malignant nature of the new growths was indicated by their morphology and transplantability(1). The sarcomas induced by 20-methyl-cholanthrene were similar to those reported with benzanthracene; it was noted, however, that often they originated as early as the 60th day. This rapid induction was a favorable condition in which the earliest stages of sarcoma inception might be studied.

Experimental. Tumors were produced in 6 hamsters by the injection subcutaneously into the right median thigh area of 0.5 mg of 20-methylcholanthrene in 0.25 ml of tricaprylin. As soon as the first tumor appeared, trocar transplants were made in 7 animals. Thirteen days later all of the injected animals showed small tumors. Transplants were carried through 5 generations. Twelve animals were used in each generation of transplants. In the first generation, 11 takes were recorded; 10 in the second generation, 7 in the third, 9 in the fourth and 9 in the fifth. Growth of the transplants in the last series was evident within one week following transplantation.

Following this pilot experiment, groups of animals were injected subcutaneously with 1 mg 20-methylcholanthrene in: (1) olive oil plus carbon black, (2) saline suspension plus carbon black, (3) saline suspension. Twenty animals were injected in each group. In Group I, there were 19 animals which subsequently showed tumors. In Groups II and III, no tumors were found. Following in-

* Doctor Smith's present address is Department of Pathology, Duke University, School of Medicine, Durham, N.C.

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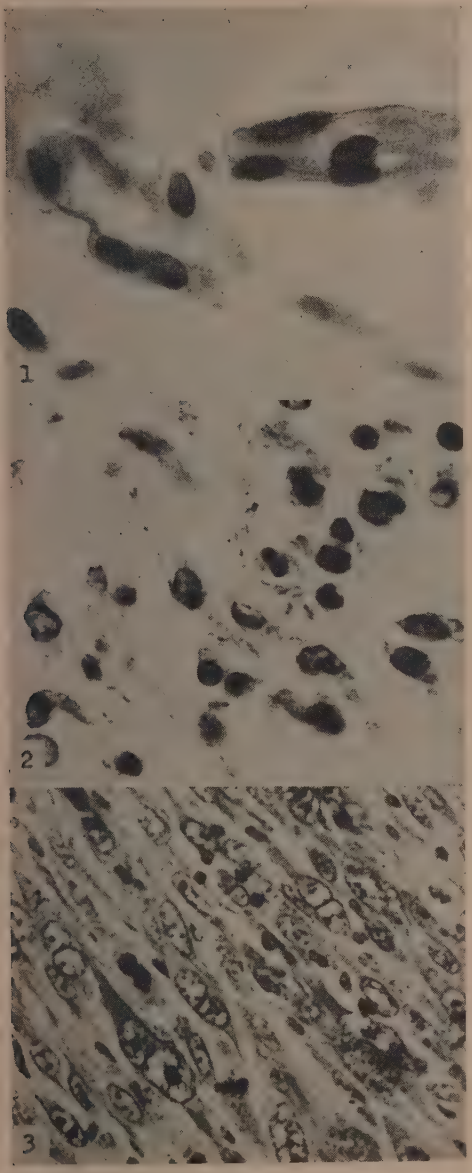

FIG. 1.

This photomicrograph of a 48 hour injection site shows 4 cells characteristic of the alteration which was first found at 24 hours and which persists until frank sarcoma supervenes. Note the massive edema spaces, blunt ended cells on right and tailed ones on left. The cytoplasm is swollen, hyperchromatic. The nuclei are very hyperchromatic. All sections —H. & E. stain.

FIG. 2.

A 4 day injection site showing connective tissue culture like arrangement of fibroblasts. Tailed and blunt cells and a few inflammatory cells are seen. Edema is still marked. H. & E. stain.

FIG. 3.

High magnification of one of the fibrosarcomas. Compare the large nucleated cells to the cell described in Fig. 2. The edema has disappeared and sheets of cells are seen.

jection animals were sacrificed in each group at 24, 48 hours; 3, 4, 7, 10 days and at 2, 3, and 4 weeks.

In the early part of the experiments, before neoplasia was apparent, tissue was removed from the site of the carbon black. Grossly, except for edema, there was little change at the injection sites. Small oil droplets of the injected solutions were found and after 3 weeks small areas of fibrosis could be discerned. Microscopically, however, striking tissue changes were found. Within 24 hours there was edema of the connective tissue, and within 48 hours these cells showed cytoplasmic granularity. The connective tissue cells, in the areas of injection, showed an alteration which was to remain constant in all sections studied until frank sarcoma was established. This alteration consisted of swelling and granularity of the cytoplasm with hyperchromatism. The nuclei were enlarged, very hyperchromatic, and either solid blue or very hydropic (Fig. 1 and 2). There was a marked edema which persisted until the end of the first week. A scattering of inflammatory cells were present, but there was little cell necrosis. The altered connective tissue cells were separated into single cells or small groups and were blunt ended or had long cytoplasmic tails. The groups of changed connective tissue cells were studied from the standpoint of their origin. They simulate macrophages but never, at any stage, did they contain carbon black. The capillary endothelial cells were not affected. In fact, the only cells which showed striking and constant cytoplasmic and nuclear alteration were the connective tissue cells. These cells within a brief period of 4 days were already forming into small sheets which simulated minute tissue cultures. After one week, the areas condensed into small nodules, which lay next to

the oil droplets. In these areas, macrophages filled with carbon black were readily distinguishable from sheets of connective tissue cells. Cell necrosis was never prominent following oil injections. In contrast, the areas in which 20-methylcholanthrene was injected in saline suspension showed early severe cell necrosis. At 3 days there was necrosis of connective and adipose tissues with cell alterations similar to, but not as intense as, those seen surrounding fat solutions. Many tissue slits were found in which the crystals had been dissolved and giant cell formation was found. The necrosis became more extensive up to one week, and by ten days the process appeared static. In oil injection, poor granulation tissue formed, and by the third week, microscopically large sheets of abnormal connective tissue cells have formed. Within 8 to 10 weeks from these sites frank sarcomas grew (Fig. 3). As nearly as can be ascertained from studying sections from different animals in the series at the sites there are stages of cell proliferation followed by regression. The whole cycle is repeated a number of times before the true infiltrative sarcoma is finally established.

Discussion. Most investigations on tumor induction have studied the tumor after it became established. By marking the site with carbon black, we were able to study the cell alterations at an extremely early time. With this technic it became clear that carcinogen produced a distinctive cell alteration which is established within 72 hours following the injection and that this alteration was neither extensively destructive to the cell nor directly stimulatory to cell growth. Wolbach(2) in studies on skin arrived at a similar conclusion for that tissue. The changes induced *in vivo* are very similar to those that had previously been found to occur when connective tissue cultures are exposed to a carcinogen(3). It was surprising to find this distinctive cell alteration taking place so long before invasive sarcoma finally is found. Wolbach(4) further found that the carcinogens kept the tissue from healing. There was a cut down in reparative processes. We noticed a similar finding and the same kind of phenomenon has been noted in tissue cultures by Voegtlin(5), who found a striking growth inhibition in methylcholanthrene exposed cultures.

A variety of special stains were done on the established tumors in order to try to specify their origin. The results of these special stains were often equivocal. With the technic of marking the macrophages with carbon black and studying the other tissue components, it was clear that from the beginning the cells which finally grew into the fibrosarcoma originated from the connective tissue fibroblasts. Orr(6) who did a study of tissues removed somewhat later than ours noticed that formation of fibrous connective tissue was deficient at the site of carcinogen injection, but did not study the cell race of origin.

The edema which is noted during the first week is excessive and finally disappears; this is out of proportion to the very scant amount of inflammatory exudate in the lipoid injected sites. Following the edema phase, the altered connective tissue cells grow in a small locus in fairly close proximity to the injected areas. The lack of cell necrosis would seem to rule it out as an essential characteristic of the induction process.

Summary. The subcutaneous injection of 20-methylcholanthrene into hamsters produced fibrosarcomas within 60 to 80 days. Examination of the injection sites, 24 hours following injection, showed fibroblasts which are morphologically different from normal. At first these cells show little active proliferation. The altered fibroblasts form a distinct strain of cells in a nodulus close to the area of injection and it is from these nodules of altered fibroblasts that invasive fibrosarcoma grows. In contrast to the oil solutions of carcinogen used, the saline suspensions produce cell necrosis and giant cell reaction.

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Paper Chromatography of Protein Mixtures and Blood Plasmas. (18055)

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It has been shown by Franklin and Quastel (1) that it is possible, by means of paper chromatography, to effect separations of proteins on filter paper, using the capillary ascent technic (Williams & Kirby)(2) and hemin as a 'marker' for the proteins with which it combines. Buffer and aqueous salt solutions, instead of nonaqueous solvents, are employed as developing agents. Working with solutions of pure (crystallized) proteins these workers have shown that the results of two dimensional paper chromatography are in accordance with the findings of electrophoretic analyses, those proteins exhibiting homogeneity or heterogeneity under the conditions of electrophoresis giving substantially the same patterns on filter paper. Moreover, they have shown that a protein such as egg albumin, does not lose its serological properties (precipitin formation with the specific antiserum) on its ascent on filter paper, and that an enzyme such as urease, may ascend filter paper to a particular position without loss of its enzyme properties. It is evident, therefore, that the technic may be applied to the separation, and possible identification, of proteins and enzymes. It is intended, in a series of forthcoming papers, to show how the technic, modified from that originally described by Franklin and Quastel, may be applied to the study of animal sera and plasmas under different pathological conditions and to the investigations of various problems relating to the interaction of proteins, enzymes and a variety of organic substances.

It has already been demonstrated by Gross, Leblond, Franklin and Quastel(3) that the technic of paper chromatography may be used for showing the combinations between thyroxine and plasma proteins.

It is of interest, in connection with this work, that Cochran(4) has already shown that the protein of tobacco mosaic virus will ascend filter paper and still retain its infectivity; incidentally, its position on paper was marked by the application of the Sakaguchi test for arginine. More recently, Mitchell, Gordon and Haskins(5) have succeeded in separating constituents of takadiastase by the use of a chromatopile.

It is intended, in this paper, to give a brief account of the chromatography of protein mixtures with particular reference to animal plasmas.

Procedure. Whatman No. 1 filter paper is used almost exclusively throughout this work, although other filter papers have been tried. The paper is cut into 8″ x 8″ squares, and the aliquot of protein solution is placed in the lower left hand corner. Plasmas are taken with a heparinized syringe (1/10 ml of 1% heparin-Na salt for 10 ml blood), and centrifuged for about 20 minutes. The plasma is placed in culture tubes in 0.5 ml quantities, and 0.02 ml 0.3% hemin is added. If a surface active agent is to be used, it is generally added to the tube first to facilitate its solution. The contents of the tube are now well mixed, and 0.02 ml aliquots are applied to the paper, each sample usually being tested in duplicate. After the aliquot has dried at room temperature, the paper is formed into a cylinder in such a manner that the edges do not touch. The cylinders are placed upright in a pyrex plate, which contains 100 ml of the developing solution. It is covered with a suitable container (a large

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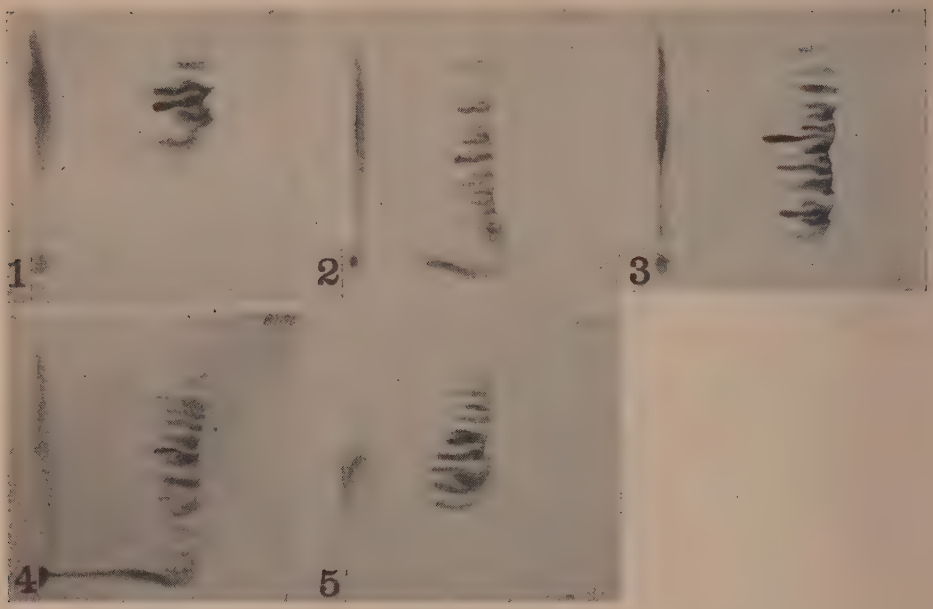

FIG. 1. Human plasma.
FIG. 2. Human plasma with 0.03 ml "Span 20" added per 0.5 ml plasma.
FIG. 3. Human plasma with 0.03 ml "NNO" added per 0.5 ml plasma.
FIG. 4. Human plasma with 0.05 ml "BRIJ-30" per 0.5 ml plasma.
FIG. 5. Human plasma with 0.04 ml "Tween 20" per 0.5 ml plasma.

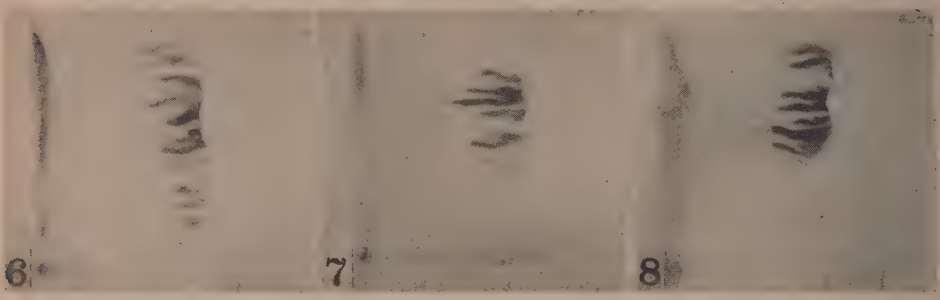

FIG. 6. Human plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.
FIG. 7. Rat plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.
FIG. 8. Guinea pig plasma with 5 mg "Elvanol 31-31" added per 0.5 ml plasma.

cylinder) to maintain constant humidity. After the solution has reached the top of the cylinder, which usually requires about 90 minutes, the cylinder is taken out and allowed to dry at room temperature. After thorough drying, the cylinder is re-formed and stapled again at right angles to the original direction and placed in the pyrex dish which contains the developing solution required for the second dimension. It is not necessary to allow the solution to reach the top of the filter paper in the second dimension, but it may be stopped about two thirds of the way up. The cylinder is again dried, and the filter paper is streaked with freshly prepared benzidine reagent. The chromatogram is photographed immediately due to the rather rapid background color development. The entire process may be easily carried out in 5-6 hours.

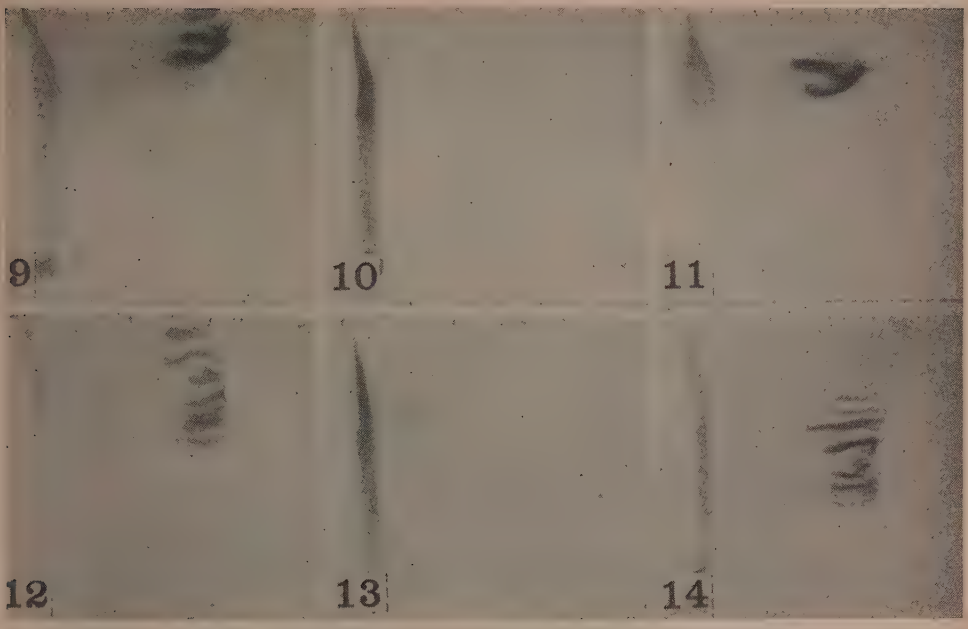

FIG. 9. Protein (Cohn) Fraction V (16 mg) dissolved in 0.5 ml 0.85% saline.
FIG. 10. Protein (Cohn) Fraction III (8 mg) dissolved in 1.0 ml 0.85% saline.
FIG. 11. Mixture of Protein (Cohn) Fractions; 22 mg V + 4 mg II + 5 mg IV—3,4, dissolved in 0.5 ml 0.85% saline.
FIG. 12. As in Fig. 9 with 0.01 ml "Tween 85" per 0.5 ml solution.
FIG. 13. As in Fig. 10 with 0.01 ml "Tween 85" per 0.5 ml solution.
Fig. 14. As in Fig. 11 with 0.01 ml "Tween 85" per 0.5 ml solution.

Hemin. The hemin solution is prepared by dissolving 30 mg crystalline hemin in 10 ml of distilled water, to which has been added a small quantity of sodium bicarbonate. Hemin solution is generally added in the ratio of 0.02 ml hemin to 0.5 ml of the protein solution.

Benzidine solution. This solution must be prepared daily, by mixing equal volumes of saturated alcoholic benzidine solution and 3% hydrogen peroxide. It is made acid with glacial acetic acid. The solution is applied with a paint brush, and the chromatogram is photographed immediately.

Developing solutions. A large number of solutions of organic salts and buffers at different concentrations have been investigated for their suitability as developing agents. It has been found that M/10 sucrose solution is very efficient for use in the first dimension, when this is followed by M/10 sodium potassium tartrate solution in the second dimension. This combination is used in all the chromatograms given in this paper (Fig. 1-18). Other combinations also give very good results, as for example, dextrose, lactose or maltose in the first dimension, and nicotinate, malate, succinate, fumarate, mandelate, acetate or citrate in the second dimension. Certain amino-acids also give good results when employed in developing solutions, for example, M/10 methionine solution followed by M/10 acetyl glycine solution, or M/10 cysteine solution followed by M/10 sodium glutamate solution.

Effects of various filter papers. The choice of filter papers seems to be very important, as it appears that not all animal plasmas give the best results with the same filter paper. Human and rat plasmas give the best chromatograms with Whatman No. 1 but guinea pig plasma gives the best results when

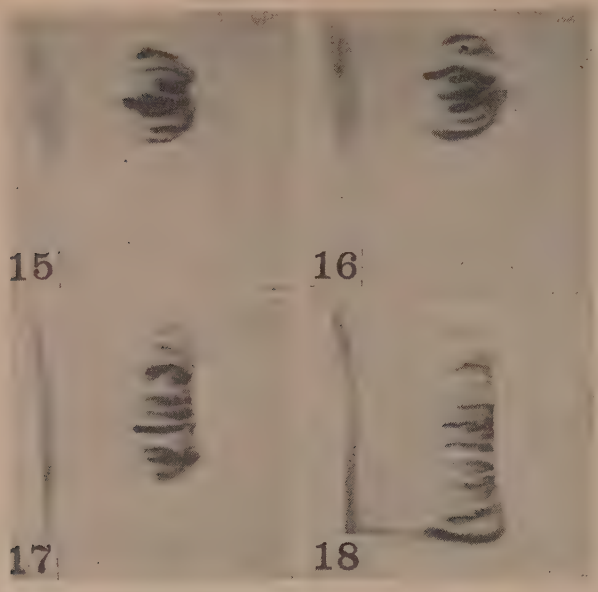

FIG. 15. Normal horse serum.
FIG. 16. Horse serum immunized against diphtheria toxin.
FIG. 17. As in Fig. 15 with 0.01 ml "Tween 85" per 0.5 ml serum.
FIG. 18. As in Fig. 16 with 0.01 ml "Tween 85" per 0.5 ml serum.

using S and S 589. When comparing the plasmas of various species, however, Whatman No. 1 has been consistently used.

Effects of the addition of surface active substances. When a plasma is chromatographed in the manner that has been described above, the various protein components do not separate very well and tend to remain bunched together. This is illustrated in Fig. 1. It is found that if certain surface active substances are added to the plasma before chromatography, good separations of the protein fractions take place. The following substances yield excellent results: sodium alginate, the "Elvanols," "Spans," "Tweens," "BRIJ" and "NNO."* Results with sodium tauroglycholate are relatively poor. The various surface active substances do not give identical results, both their structures and their concentrations being important in determining the pattern of the protein chromatogram. It has been made a matter of practice to make chromatograms of a blood plasma, or a protein mixture, in presence of different concentrations of a selected surface active substance. Usually 0.01 ml to 0.06 ml of the solution of the surface active agent is added to 0.5 ml plasma before addition of the hemin, and subsequent chromatography. The separation of the plasma constituents, as facilitated by the presence of surface active agents, are shown in Fig. 2, 3, 4 and 5. High concentrations of surface active agents must not be added to the plasma; in their presence, the movement of the proteins on filter paper appears to be greatly suppressed.

Effects of dialysis of blood plasma. Dialysis of blood plasma against running water seems not to disturb the pattern of the

*"Elvanols": A group of polyvinyl alcohols produced by hydrolysis of polyvinyl acetates of varying degrees of polymerization (Dupont).
Span 20: Sorbitan monolaurate.
NNO: Glycerol mannitan laurate.
BRIJ 30: Polyoxyethylene lauryl alcohol.
Tween 20: Polyoxyethylene sorbitan monolaurate.
Tween 85: Polyoxyethylene sorbitan trioleate.

plasma chromatogram. It is concluded, therefore, that the dialysable material of plasma has little or no influence on the movement of the proteins on filter paper.

Consistency of results. With any given plasma, or protein mixture, excellent duplication of results has been obtained. It is necessary to be strictly accurate in the measurement of the surface active material added to the plasma, but if due care is taken, there is no difficulty in obtaining good duplication with any sample of a protein mixture.

Applications of the technic. Comparison of human, rat and guinea pig plasmas. Heparinized plasmas of different animals show different patterns on the chromatogram when they are chromatographed under similar conditions.

With rat and guinea pig, the blood is removed with a heparinized syringe from the ether anaesthetized animal, and centrifuged for about 20 minutes. 0.5 ml of plasma is mixed with 0.02 ml 0.3% hemin solution, and various amounts of a surface active agent is added to a number of such samples. Using 5 mg "Elvanol 31-31" as surface active agent and two dimensional chromatography with M/10 sucrose solution in the first dimension followed by M/10 sodium potassium tartrate solution in the second, evident differences between the plasma chromatograms appear (Fig. 6, 7, and 8). On the other hand, the presence of "Elvanol 51-05" fails to show any obvious differences. "Elvanol 54-22" yields a much better separation of constituents of human plasma than is obtained with either rat or guinea pig plasma. "Span 20" gives excellent separations of the protein components, but, whilst failing to show a marked difference between human and rat plasma, it gives a distinctive pattern with guinea pig plasma. "NNO" gives a good separation of the constituents of each of the plasmas, but differences between the plasma patterns are not very evident. "Tween 20," "Tween 81" and "Tween 85" are the best surface active agents to use when examining various human plasmas.

As a matter of practice when chromatographing human plasmas, from different pathological conditions, "Tween 81" and "Tween 85" are being used regularly. They seem to give the most satisfactory separations so far. It is obvious, however, that there is great room for improvement, and new surface active agents are being continually tested.

It has already been found that the pattern of the chromatogram of a plasma, depends on the nutrition of the animal and the results of our investigation into this matter will form the subject of a separate communication. It is desirable, in comparing the plasmas of patients, to keep dietary conditions as constant as possible. Preliminary work with the plasmas of dogs, maintained on a selected diet, has shown little individual variation, so long as a proper control of the diet is maintained.

Chromatography of pure proteins and protein mixtures. A number of chromatograms has been made of preparations of purified proteins and protein fractions isolated from blood. These preparations have been kindly presented by the courtesy of the Cutter Laboratories and consist of the fractions of blood plasma isolated according to the procedures of Dr. Cohn and his colleagues(6). Electrophoretic analyses have shown that fraction III consists of a mixture of α, β, and γ globulins and that of fraction V is largely albumin. Chromatograms of fraction V are shown in Fig. 9 (without Tween) and Fig. 12 (with Tween) and those of fraction of III are shown in Fig. 10 (without Tween) and in Fig. 13 (with Tween). It will be noted that the chromatograms of fraction V indicate the presence of more than one protein, whilst those of fraction III show but little movement in the second dimension. The chromatograms of the separate fractions do not resemble that of blood plasma. When mixtures of the fractions are taken in such proportions and in such quantities as to constitute the proportions and amounts of albumin and globulin in blood plasma, chromatograms are obtained which resemble those of normal blood plasma.

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This is shown in Fig. 11 (without Tween) and Fig. 14 (with Tween). The evidence from a number of chromatograms of protein mixtures indicates that an interaction between proteins occurs, whereby the chromatogram of a mixture of proteins is not necessarily that to be expected from a simple addition of the chromatograms of the proteins taken separately.

This fact is also illustrated by the results of some preliminary experiments carried out with mixtures of proteins with hemoglobin and with cytochrome *C*. Chromatograms indicate that both these substances may form associations with other proteins, but further experiments are required to decide whether the conditions of the chromatographic technic may result in sufficient dissociation of the hemin from the hemoglobin or from the cytochrome to produce hemin complexes with the other proteins present.

Comparison of normal and hyper-immunized horse serum. Through the courtesy of the Department of Microbiology and Hygiene of the University of Montreal, specimens of normal horse sera and the sera of hyperimmunized horses (immunized against diphtheria toxin) have been obtained. Chromatograms of the two sera both with and without the addition of a surface active agent are shown in Fig. 15, 16, 17, and 18. For experiment, 0.01 ml "Tween 85" was added to a mixture of 0.02 ml 0.3% hemin solution and 0.5 ml serum. Chromatography was carried out on Whatman No. 1 filter paper using M/10 sucrose solution in the first dimension and M/10 sodium potassium tartrate solution in the second dimension. It will be seen that the chromatograms of the sera (both with and without the addition of the Tween) show differences from each other. That of the hyperimmunized serum indicates the existence of fractions not present in the normal serum.

Summary. A technic for the two dimensional chromatography of blood plasma and protein mixtures on filter paper is described. Separations of protein constituents are greatly facilitated by the addition of surface active substances such as the "Tweens" or "Spans," etc. The technic at present adopted is to add "Tween 85" or "Tween 81" and, using hemin as protein marker, to employ M/10 sucrose solution in the first dimension and M/10 sodium potassium tartrate solution in the second dimension. Differences between the protein patterns of chromatograms of human, rat and guinea pig plasma are noted. With protein mixtures, associations of proteins may occur that may be detected by chromatography. The sera of hyperimmunized horses (as against diphtheria toxin) yield chromatograms which differ from, and exhibit fractions not present in, chromatograms of normal horse sera.

Grateful acknowledgment is made to the National Cancer Institute of Canada for a grant in aid of this work, to the Department of Microbiology and Hygiene of the University of Montreal for gifts of normal and immune sera, to Dr. J. Gross for the protein fractions obtained from the Cutter Laboratories, Berkeley, Calif., to the Atlas Powder Co. (per G. F. Sterne & Son, Brantford, Ont.), for gifts of "Spans," "Tweens," "NNO," and "BRIJ-30," to the Canadian Industries, Ltd., Montreal, for gifts of "Elvanols," and to Mrs. Mary Hersey for her able assistance in this work.

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Comparative Study of Morphine and Dromoran as Antidiuretic Agents in the Dog.* (18056)

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The influence of morphine on renal function has been the subject of investigation for many years. Experimental results have not been conclusive and controversy still exists regarding the exact mechanisms involved.

Stimulated by earlier clinical observations and speculations, Fee(1) noted that morphine inhibited water diuresis in decorticated and decerebrated dogs and cats. Stehle and Bourne(2) studied the effects of morphine on kidney function in dogs with bladder fistulae. They observed minimal anti-diuretic effects of morphine when urinary flows were normal or low, however, in the presence of high urinary flows morphine exerted a marked anti-diuretic effect. Chloride excretion was also studied and in most cases chloride excretion was reduced by morphine although the results were variable.

Fee(3) employed himself as an experimental subject and noted that the administration of morphine produced an anti-diuretic effect accompanied by chloruresis. Having eliminated cortical and cerebral effects in his previous experiments in the dog, he concluded that the posterior pituitary gland was responsible for regulation of the renal excretion of water and chlorides. It remained for Ranson and his co-workers(4) to definitely establish this concept.

The nature and mechanism of morphine effect on renal function was further investigated by De Bodo(5,6,7) in normal and diabetes insipidus dogs. He concluded that morphine owed its anti-diuretic and chloruretic effects to stimulation of the neurohypophysis with release of the anti-diuretic hormone.

Handley(8) recently studied changes in renal hemodynamics produced by morphine and reported a reduction in glomerular filtration rate, renal plasma flow, and Tm_G both in normal and in diabetes insipidus dogs. He concluded that morphine could reduce urine output by either reducing the number of active nephrons or by causing the liberation of anti-diuretic hormone.

Clinical reports on the anti-diuretic effects of morphine have been less decisive. Boyd and Scherf(9) found that although morphine was anti-diuretic in the normal human, it caused diuresis presumably through extra-renal factors in patients with cardiac decompensation. Ferrer and Sokoloff(10) confirmed the anti-diuretic and chloruretic action of morphine in normal humans, but noted anti-diuresis with reduced chloride excretion in 3 out of 9 cardiac patients receiving mercurial diuretics in addition to morphine. More recently, Kraushaar *et al.*(11) reported that although morphine was anti-diuretic in pregnant and non-pregnant women, it caused a

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reduction of urinary chloride excretion in 4 out of 6 patients. In contrast to morphine, pitressin produced chloruresis in this series. Since morphine produced an anti-diuretic effect in 2 patients with clinical diabetes insipidus, although to a lesser degree than in normals, the authors concluded that morphine anti-diuresis was not mediated through the stimulation of the neuro-hypophysis in man. Brown, Hodges and Bradbury(12) observed that in the human, morphine effected a reduction in renal plasma flow, did not affect glomerular filtration rate, and increased the tubular resorption of water.

The anti-diuretic properties of morphine may be undesirable in clinical conditions of oliguria and edema associated with salt and water retention. Recently, a morphine substitute, 3-hydroxy-N-methyl morphinan hydrobromide (Dromoran†) has been reported to be an effective analgesic with fewer side reactions than morphine(13,14,15). The purpose of this investigation was to study the possible anti-diuretic effects of Dromoran in the dog and to compare its renotropic effects with those of morphine.

Methods. All experiments were performed on trained, unanesthetized female dogs in the fasting state. Essentially the same procedures as reported by De Bodo(5) were used. Each experiment consisted of 2 consecutive diuresis periods of 3 hours duration. The animals were hydrated with tapwater (40 cc/kg) by stomach tube at the start of each period. Urine samples were collected by means of indwelling urethral catheters. Total urine volumes for each period were recorded and aliquots were taken for chloride analysis which were done by the method of Schales(16). Control runs were made to determine the normal diuretic response to the doses of water administered in Periods I and II.

TABLE I.
Control Diuretic Responses Following Second Hydration During Period II.

Dog	Hydration vol. (total cc)	Urine vol. (cc/3 hr)	% retention
Ev.	920	790	13.7
Br.	800	720	10.0
Tr.	650	550	15.3
Qu.	650	576	11.4
Co.	640	555	13.3
Sp.	800	615	23.1
Mean		—	14.5

All drugs were administered during Period II. Since morphine occasionally produced emesis, it was routinely injected 40 minutes after the second hydration which initiated Period II. In this way, no appreciable loss of the hydration dose occurred, since complete absorption of this dose of water has been shown to occur within a 40 minute period(5). The volume of urine output during Period II was divided by the hydration volume to obtain the per cent of water returned. Any anti-diuretic effect was calculated by arbitrarily subtracting the per cent of water return from the ideal return of 100%.

Morphine sulphate was administered subcutaneously in doses of 0.5, 0.75, 1.0, 2.5 and 5.0 mg/kg. Dromoran was injected subcutaneously in doses of 0.25, 0.5 and 1.0 mg/kg. The responses to these doses were plotted to obtain the anti-diuretic dose which would produce 50% inhibition of water diuresis (AD_{50}).

Results. Table I summarizes the diuretic responses following hydration in the dog under controlled conditions. In Period II approximately 85% of the administered water was excreted, yielding an average "inhibition" of 15%. These data compare favorably with those of De Bodo. The anti-diuretic responses to increasing doses of Dromoran and morphine are presented in Table II. It is evident that both drugs are anti-diuretic in the doses employed, although the variability

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† Dromoran Hydrobromide is the trademark of this compound which is also reported in the literature as "Nu-2206."

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TABLE II.
Effect of Morphine and Dromoran on Water Diuresis in the Normal Dog.

Drug	Dose, mg/kg s.c.	No. of dogs	% inhibition of water diuresis (mean)	Standard deviation*
Morphine	5.0	4	66.5	8.44
	2.5	6	66.1	15.7
	1.0	7	52.9	13.3
	0.75	5	42.2	8.07
	0.5	5	17.8	7.30
Dromoran	1.0	5	60.4	8.71
	0.5	5	60.6	7.0
	0.25	5	30.9	17.1

* Estimated from range/σ.

TABLE III.
Effects of Water Diuresis on Chloride Excretion.

Dog	Total urinary chlorides (milliequivalents)			Relative chloride concentration (milliequivalents/liter)		
	Period I	Period II	Difference	Period I	Period II	Difference
Co.	1.47	1.06	0.41	8.14	3.41	4.73
Qu.	3.22	1.61	1.61	5.89	3.20	2.69
Za.	0.75	0.72	0.03	2.98	1.33	1.65
Sh.	2.46	0.85	1.61	8.47	1.71	6.76
Br.	0.88	0.53	0.35	1.97	1.48	0.49
Mean difference			0.80			5.26
Probability value (P)			0.05			0.08

TABLE IV.
Effects of Morphine and Dromoran on Urinary Chloride Excretion During Water Diuresis in the Dog.

Drug	Dose, mg/kg s.c.	No. of animals	Mean decrease in urinary chloride excretion			
			Total meq.	S.D.	Meq./l	S.D.
Control	—	5	0.80	0.75	3.26	2.65
Dromoran	0.25	4	1.35	1.72	2.84	2.85
	0.50	5	1.88	1.86	2.22	2.64
	1.00	5	2.74	1.55	3.86	2.74
Morphine	0.50	5	0.93	0.86	2.22	2.12
	0.75	5	4.50	3.59	13.55	16.16
	1.0	4	1.45	1.56	3.39	4.34
	2.50	5	1.52	1.45	1.92	4.09

in the results is quite marked. The AD_{50} dose for morphine was 0.9 mg/kg and for Dromoran, 0.4 mg/kg. The decrease in chloride excretion caused by hydration under controlled conditions is summarized in Table III. The difference between Periods I and II in terms of total milliequivalents of urinary chloride was statistically significant. The differences in relative chloride concentrations in meq./liter between Periods I and II just exceeded the probability level of statistical significance. In view of the variable effects of water diuresis on chloride excretion, had more experiments been run, the 5% probability level would, in all likelihood, have been satisfied insofar as the relative chloride concentration was concerned. The effects of morphine and Dromoran on chloride excretion are tabulated in Table IV. It is apparent that neither drug effected chloruresis and that the decrease in chloride excretion in Period II is probably a hydration phenomenon.

During the course of the experiments the

side-effects of the two drugs were noted. Animals receiving Dromoran showed a lower incidence of emesis and diarrhea than with morphine.

Summary. The results indicate that Dromoran, as well as morphine, has anti-diuretic activity in the range of doses studied. Comparison of the relative anti-diuretic potencies of the two drugs must of necessity take into consideration their analgesic potencies. Clinical studies indicate that Dromoran is approximately 4 times as potent an analgesic as morphine in the human(15). Since no data are available on Dromoran's analgesic potency in the dog, the AD_{50} doses of Dromoran and morphine obtained in the dog were arbitrarily related to the analgesic potency of these drugs in man.

If the following ratio is computed:

$$\frac{\text{Equivalent analgesic dose (man)}}{AD_{50}\text{ (dog)}} = \text{Anti-diuretic index (A.D.I.)}$$

it is apparent that for equivalent analgesic effects, the larger the dose required to produce 50% inhibition of water diuresis in the dog, the less the anti-diuretic potency. The quotient of this ratio can be arbitrarily termed an anti-diuretic index (A.D.I.)

$$\text{For morphine } \frac{1}{0.9} = 1.1 \text{ (A.D.I.)}$$

$$\text{For Dromoran } \frac{0.25}{0.40} = 0.6 \text{ (A.D.I.)}$$

At equivalent analgesic doses, it is obvious that Dromoran has a lower anti-diuretic index than morphine.

A review of the literature indicated no dose-response relationships in reference to chloruretic responses to morphine. De Bodo obtained chloruresis in the dog with doses larger than those which we employed. Ferrer and Sokoloff noted chloruresis in 2 human patients using a dose of morphine lower than that used by Kraushaar who reported a depression of chloride excretion by morphine in 4 of 6 patients.

The data reported in this paper do not elucidate the mechanisms involved in the anti-diuretic responses. The inhibition of chloride excretion following the second hydration is consistent with inhibition of the posterior lobe during water diuresis. Neither morphine nor Dromoran effected this inhibition of anti-diuretic hormone secretion. The absence of chloruresis in the presence of anti-diuresis suggests that in the doses used, these drugs produce an anti-diuretic effect through a mechanism other than the release of anti-diuretic hormone from the posterior pituitary.

Conclusions. 1. Dromoran is less anti-diuretic than morphine when anti-diuretic activity is related to analgesic potency. 2. In the range of doses employed in these studies, neither morphine nor Dromoran effected a chloruresis. 3. The lack of chloruresis in the presence of anti-diuretic action suggests that these drugs do not act by liberation of the anti-diuretic hormone.

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Dual Antibody Response to Coxsackie and Poliomyelitis Viruses in Patients with Paralytic Poliomyelitis.* (18057)

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Simultaneous excretion of both Coxsackie (C) and poliomyelitis viruses from the intestinal tract of man during both periods of illness and apparent health(1-3), as well as the simultaneous recovery of these two viruses from flies trapped in poliomyelitis areas(4),

* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Melnick, J. L., Ledinko, N., Kaplan, A. S., and Kraft, L. M., *J. Exp. Med.*, 1950, v91, 185.

has made it important to establish whether the host is infected by both agents or whether one virus is merely in passive transit through the alimentary tract. This answer is needed before one can consider whether the symptoms of the patient represent a single or a combined infection. Patients excreting both viruses have been shown (1) to develop antibodies to the strain of C virus isolated from their stools, as well as to a homotypic (Ohio) strain. In this report we wish to present evidence that paralytic patients excreting both viruses respond serologically with antibodies to both viruses.

In the course of an investigation of the severe 1949 poliomyelitis epidemic in Easton, Pennsylvania, samples were collected from 48 consecutive patients entering the hospital for poliomyelitis.† From these we isolated *both* poliomyelitis virus and C virus from 15 patients. Two paralytic patients were selected for more detailed study.

Patient I, a 3-year-old girl, became ill on August 30 with sore throat and pharyngitis. She developed paralysis of the abdominal muscles and was admitted to the hospital on September 1. The cerebrospinal fluid contained 52 cells per cmm. She made an uneventful recovery. Both C and poliomyelitis viruses were isolated from a pool of stools collected on September 5, 6 and 7. C virus could not be recovered from throat swabs taken on September 1, 2 and 3, nor from a late stool sample of October 12. Serum was obtained for antibody tests on September 1 and again on September 30.

Patient II, a 5-year-old girl, became ill on August 30 with nuchal rigidity and weakness of the left leg. She soon developed paralysis of the left leg and weakness of the right leg and abdominal muscles. She was hospitalized on September 2. A spinal fluid examination was not done. On April 12, 1950, her left leg was still paralyzed. Both viruses were isolated from a pool of fecal specimens collected on September 3, 7 and 10. C virus was again isolated from a sample of stool collected October 4. It is worth noting that the infective titer of the stools in newborn mice fell from $10^{-3.0}$ in the acute phase specimens to $10^{-1.0}$ in the late sample. Throat swabs collected on September 2, 3 and 5 gave negative tests for C virus. Serum was obtained on September 2 and again on October 2.

The virus-containing stools of each patient were found to contain poliomyelitis virus in a titer of 10^{-2} in monkeys. This titer enabled us to use this original human source of material as the virus in the neutralization test. The C virus titer of the two virus pools in newborn mice was $10^{-1.0}$ and $10^{-3.0}$, respectively. For the C virus, the original human stool virus as well as mouse passage virus was used in neutralization tests. Sera were used in serial threefold dilutions.

The tests were set up by mixing an 18,000 r.p.m. supernate of the stool (10^{-1} concentration) with an equal volume of serum at the dilutions indicated in Table I. After an incubation period of 2 hours at room temperature, each serum-virus mixture was inoculated into 3 monkeys to test for poliomyelitis virus antibodies and into at least 8 newborn mice to test for C virus antibodies. With mouse-passage C virus, 100 ID_{50} doses were added to the serum dilutions. The highest serum dilution of Patient I which neutralized C virus on the third day of the disease was 1:10, by the thirty-second day this titer had been increased to 1:90. Calculated on the basis of that serum dilution which protects 50% of the animals, the respective titers were about 1:50 and about 1:300. The tests carried out with 100 ID_{50} mouse passaged C virus showed for the convalescent serum titers of 1:50 for complete neutralization and 1:150 for 50% protection, the acute phase serum being negative.

That Patient I also responded to infection

2. Armstrong, M. P., Wilson, F. H., McLean, W. J., Silverthorne, N., Clark, E. M., Rhodes, A. J., Knowles, D. S., Ritchie, R. C., and Donohue, W. L., *Canad. J. Pub. Health*, 1950, v41, 51.

3. Dalldorf, G., *Bull. N. Y. Acad. Med.*, 1950, v26, 329.

4. Melnick, J. L., *Bull. N. Y. Acad. Med.*, 1950, v26, 342.

† We are indebted to Dr. N. W. Larkum and Miss Natalie Levin for their generous aid in collecting the clinical samples for this study.

TABLE I. Simultaneous Development of Antibodies to Poliomyelitis Virus and C Virus.

Patient	Day of disease serum collected	ID_{50} in 10% suspension of patient's stool	Final serum dilutions				Highest serum dilution neutralizing virus completely	Dil. of serum protecting 50% of animals	Neutralization of 100 ID_{50} doses of passaged C virus: Highest serum dilution neutralizing virus completely	Neutralization of 100 ID_{50} doses of passaged C virus: Dil. of serum protecting 50% of animals
			1:10	1:30	1:90	1:270				
I		1		C virus : in mice						
	3		0/8	4/15(3)			10	a50	0	0
	32		0/15	0/8	0/14	3/12(3)	90	■300	50	150
		10		Polio virus : in monkeys						
	3		0/3	2/3			10	20		
	32		0/3	0/3	0/3	0/3	270	>270		
II		100		C virus : in mice						
	4		3/18(2)	7/14(7)			0	30	0	0
	32		0/6	0/6	0/14	4/7(3)	90	230	50	110
		10		Polio virus : in monkeys						
	4		1/3	3/3			0	15		
	32		0/3	0/3	1/3	1/3	30	270		

Denominator indicates the number of animals used per serum dilution. The numerator indicates the number of animals with disease. The number in parentheses indicates the number of mice with observable paralysis. All monkeys with disease showed some degree of paralysis (mild to severe) and all sick animals had typical poliomyelitis lesions in the spinal cord. No CNS lesions were found in monkeys which failed to develop paralysis.

with poliomyelitis virus is shown by the rise in titer between the acute and convalescent phase sera. A dilution of 1:10 of the acute phase serum neutralized completely the patient's poliomyelitis virus; the convalescent phase serum was able to do this at a dilution as great as 1:270. Using a 50% protection endpoint there was an increase of titer from 1:20 to one greater than 1:270.

Comparable increases in titer developed in the serum of Patient II. C virus excreted with her stools was completely neutralized at a dilution of 1:90 by serum obtained 32 days after the onset of the disease, whereas serum collected 4 days after the onset of the disease was negative. There was a correlative increase in titer when it was calculated on a 50% protection endpoint: the acute phase serum had a titer of 1:30 whereas the convalescent phase serum had a titer of 1:230. With mouse-passage virus, the convalescent serum yielded titers of 1:50 for complete neutralization and 1:100 for 50% protection. The acute phase serum again gave a negative test.

As in the case of Patient I, the serum of Patient II also developed neutralizing antibodies against her own strain of poliomyelitis virus. At a dilution of 1:10, serum of Patient II taken on the fourth day after the onset of the illness did not completely neutralize poliomyelitis virus; however, a 1:30 dilution of the serum taken on the thirty-second day of the disease did neutralize the virus completely. On the basis of a 50% protection endpoint, the respective titers for the acute and convalescent phase sera were 1:15 and 1:270.

When the paired sera of each patient were titrated against 100 ID_{50} of the Easton-2 strain of C virus, the same results were obtained as in the tests against the mouse-passage strains obtained from Patient I and from Patient II. Inasmuch as the Easton-2 strain is antigenically indistinguishable from Dalldorf's Type I strain, it would appear that the strains isolated from both patients belong to this latter type(3,4).

On the basis of the neutralization tests reported here and the finding of both viruses

in the stools of 13 other patients in this epidemic, we believe that the failure to detect poliomyelitis virus in the stools of a paralytic patient excreting C virus should be regarded as a technical difficulty, and should not necessarily be regarded as an indication that C virus caused the lesion resulting in paralysis. For the present, and until it is proved otherwise, the patient's paralytic manifestations should be considered to result from CNS infection with poliomyelitis virus. However, the finding of dual infections in poliomyelitis appears to occur too frequently to be regarded merely as coincidence. Is it possible that infection with poliomyelitis virus would have been a mild affair in these patients had not C virus infection been superimposed on the poliomyelitis infection? Consideration is being given to the possibility that C virus infection may be one of the predisposing factors which may turn a nonparalytic into a paralytic case.

Summary. Both poliomyelitis virus and Coxsackie virus (C virus) were isolated from the acute phase stools of several paralytic patients during an epidemic of poliomyelitis which occurred in Easton, Pa., in 1949. In 2 such cases, neutralizing antibody tests were carried out with acute and convalescent serum reacting with a suspension of virus obtained directly from acute phase stools. It was found that both patients responded to their illness by simultaneously developing antibodies both to poliomyelitis virus and to C virus.

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Effect of 17-Hydroxy-11-Dehydrocorticosterone (Compound E) and of ACTH on Arthus Reaction and Antibody Formation in the Rabbit. (18058)

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(With the technical assistance of Kenneth Grow)

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Hench and his coworkers(1) reported the beneficial effects of adrenal cortical hormone, compound E, and the adrenocorticotropic hormone in patients with rheumatoid arthritis. Subsequently, encouraging therapeutic results were obtained with these hormones in the treatment of the acute phase of rheumatic fever(2,4), periarteritis nodosa(3) and disseminated lupus erythematosus(4). These 4 diseases have in common numerous clinical manifestations and similar basic pathological alterations consisting of focal necrosis of collagen and damage to the cardiovascular system(5). Rich and Gregory(6,7) have demonstrated that hypersensitivity of the Arthus type can produce periarteritis nodosa as well as cardiovascular lesions in the rabbit resembling those of rheumatic fever. Since these observations suggest that anaphylactic hypersensitivity may play a role in the pathogenesis of this group of diseases, a study of the effect of compound E and ACTH on the development of the Arthus state in the rabbit

1. Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meetings Mayo Clin.*, 1949, v24, 181.

2. Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meetings Mayo Clin.*, 1949, v24, 277.

3. Shick, R. M., Baggenstoss, A. H., and Polley, H. F., *Proc. Staff Meetings Mayo Clin.*, 1950, v25, 135.

4. Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Arch. Int. Med.*, 1950, v85, 545.

5. Rich, A. R., *Harvey Lectures*, 1946-47, v42, 106.

6. Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1943, v72, 65.

7. Rich, A. R., and Gregory, J. E., *Bull. Johns Hopkins Hosp.*, 1943, v73, 239.

was undertaken.

Methods and materials. The ACTH, obtained from Armour and Co. in the form of unsterile powder was administered to the animals in saline solution containing an equivalent of 10 mg of standard ACTH LA-1-A per ml.

Compound E was obtained as cortisone acetate from Merck and Co., Inc. in the form of unsterile crystals.* After solution in warm anhydrous acetone, these crystals were suspended in saline and most of the acetone removed by evacuation. Finally, the suspension was made up to a volume such that 1 ml of the suspension contained 10 mg of compound E.

Crystalline hen's egg albumin, the antigen employed in this study, was prepared according to the method of Kekwick and Cannan(8).

For sensitization, 24 male albino rabbits weighing approximately 2 kg each were injected intracutaneously every day for 18 days with 0.2 ml of a crystalline egg albumin solution containing 4.2 mg of egg albumin nitrogen per ml. Beginning with the first sensitizing injection, and with each succeeding one, 8 of these animals were injected intramuscularly with one dose per day of 10 mg of compound E and another 8 animals were injected intramuscularly with 10 mg of ACTH per day given in 4 doses of 2.5 mg each every 6 hours. This treatment was maintained throughout the experiment. These total daily dosages of 5 mg/kg are approximately 3.5 times those generally employed in the treatment of man (100 mg per 70 kg of body weight). The site of each inoculation of egg albumin was observed 24 hours (in some instances at shorter intervals) after injection and every reaction was measured and recorded. All of the rabbits were weighed and bled for 2-3 ml of blood from the marginal ear vein at the commencement of the experiment and on the 4, 7, 9, 11, 14 and 16th days.

The antibody content of the serum for crystalline egg albumin was determined as follows: Egg albumin was added to 0.5 ml of serum in increments of 1 or 2 μg of nitrogen until a final addition of antigen produced no further precipitation. After each addition of antigen, the sera were incubated at 37°C for 1 hour and usually allowed to remain overnight at 0-5°C and then centrifuged. Finally, after 3 to 5 days at 0-5°C, the precipitates were collected by centrifugation in the cold and washed twice with cold saline (9). The washed precipitates were dissolved in 4.0 ml of 0.1 N sodium hydroxide and the absorption determined in a 1 cm quartz cell at a wavelength of 280 millimicrons with a Beckman spectrophotometer. At this wavelength the absorption of such a solution is proportional to its antibody-antigen nitrogen content. The resulting optical densities were therefore converted into precipitate nitrogen from a standard reference curve. Since complement is not present in aged sera,† the antibody nitrogen was obtained by subtraction of antigen nitrogen from the total value. Tests on the supernatant sera were all positive for slight excess antigen. Duplicate determinations were done wherever possible. These agreed usually within $\pm$ 5% when the antibody content was above 100 μg per ml.‡

* Since there are no apparent differences in the physiologic effects of cortisone (Compound E) and cortisone acetate, the latter compound is referred to throughout as compound E.

8. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, 1936, v30, 227.

9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, v62, 697.

10. Sayers, G., and Sayers, M. A., *Ann. New York Acad. Sci.*, 1949, v50, 522.

† None of the sera tested exhibited complement activity.

‡ Only small volumes of sera were taken for analysis, to avoid possible stress on the pituitary-adrenal system(10). Therefore the standard precipitin method as devised by Heidelberger and Kendall(9) was not employed. The present method is not a precise measure of antibody content; for example, with the serial addition of antigen, "univalent antibody might remain undetected. To determine the magnitude of this error, several of the sera obtained on the final bleeding were tested by both methods. It was found that only with sera of high antibody content was there disagreement, in which case the values obtained with the standard method were substantially higher because of the coprecipitation of "univalent"

TABLE I.

Effect of Compound E and ACTH on Production of Arthus State in Rabbits Receiving Daily Intracutaneous Injections of Egg Albumin. (Figures presented below represent the product of the height, width and length of the 24-hr skin reactions measured in centimeters).

Rabbit No.	Treatment	Magnitude and appearance of 24-hr skin reactions observed on the following days:													
		5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	None														
	Control	0	0	3.9*	18 *	10 *	6.7†	—	16 ‡	—	18 ‡	7.6‡	7.4‡	14 ‡	—
2	,,	0	.3*	.3*	.7*	3.0*	2.9*	4.2*	7.6*	5.7*	4.6*	4.8*	4.7*	2.7*	2.8*
3	,,	.3*	—	.3*	.2*	1.2*	1.2*	2.1*	2.2*	4.9*	2.2*	2.2*	1.0*	2.8*	2.9*
4	,,	.3*	0	.2*	.2*	2.1*	6.6‡	22 ‡	23 ‡	—	19 ‡	9.6‡	2.6‡	12 ‡	—
5	,,	0	.6*	.7*	.9*	3.0*	13 *	14 *	30 ‡	24 ‡	33 ‡	33 ‡	31 ‡	25 ‡	24 ‡
6	,,	.4*	0	.3*	.7*	.3*	0.4*	1.4*§	4.1*§	10 ‡§	Dead				
7	,,	0	0	.6*	0	.7*	10 *	12 ‡	6.0‡	9.9‡	23 ‡	17 ‡	25 ‡	12 ‡	12 ‡
8	,,	0	0	0	.5*	.7*	47 *	68 ‡	37 ‡	39 ‡	51 ‡	32 ‡	43 ‡	40 ‡	61 ‡
9	Compound E	0	0	0	0	0	0	0	.4*	0	.2*	.6*	.4*	.2*	.7*
10	,,	0	0	0	0	0	0	0 §	.3*§	0 §	.3*	.4*	.3*	.5*	.4*
11	,,	0	0	0	0	0	.1*	0	0	0	.1*	.1*	.1*	.1*	.4*
12	,,	0	0	0	0	.1*	0	0	0	0	0	.1*	0	.1*§	.1*§
13	,,	0	0	0	0	0	0	.3*	.2*	.1*	0	.4*	.2*	0	.2*
14	,,	0	0	0 §	0 §	0	0 §	0 §	0 §	.1*§	0 §	.2*§	Dead		
15	,,	0	0	0	0	0	0	0 §	.1*	0 §	0 §	.1*§	0 §	Dead	
16	,,	0	0	0	0	0	0	0 §	0 §	0	0	0	0	Dead	
17	ACTH	0	0	0	0	4.4*	4.7*	7.2*	9.7*	7.2*	7.8*	3.5*	3.5*	1.9*	.4*
18	,,	0	0	.6*	3.1*	6.1*	8.7‡	12 ‡	25 ‡	15 ‡	25 ‡	15 ‡	14 ‡	11 ‡	13 ‡
19	,,	0	0	0	0	0.1*	0	0	0	0	.3*	.3*	.2*	.4*	.1*
21	,,	0 §	0 §	0 §	0 §	0 §	0 §	0 §	0 §	.6*§	2.0*§	Killed			
22	,,	0	0	0	.9*	3.8*	10 *	8.4†	5.1‡	8.8‡	22 ‡	34 ‡	10 ‡	19 ‡	15 ‡
23	,,	0	0	0	0	3.4*	6.7†	9.2‡	8.4‡	3.0‡	10 ‡	12 ‡	7 ‡	8 ‡	6 ‡
24	,,	0	0	0	0	4.2*	.9*	5.6*	.8*	0	3.3*	.9*	1.1*	.5*	1.9*

* Pink. † Red. ‡ Hemorrhagic. § Diarrhea.

Most of the sera obtained from the animals treated with compound E were lipemic. Because of the possibility that this alteration might in some manner affect the reactivity of the antibody with antigen, the ability of the serum to passively sensitize guinea pigs was determined. Guinea pigs weighing approximately 250 g were injected intravenously using the hind leg vein with varying amounts of rabbit serum obtained from the final bleeding. Two days later, these animals were challenged with a shocking dose of egg albumin (1 mg in 1 ml saline) and their reactions noted.

On the 18th day, *passive* Arthus reactions were induced in all of the animals(11). The rabbits were injected intracutaneously at two different sites with 0.4 ml of antipneumococcus type II rabbit antiserum representing 1 mg and 0.5 mg of antibody nitrogen respectively. One-half hour later, 1 mg of pneumococcus type II polysaccharide was injected into each site. Skin lesions were observed and recorded 8 and 24 hours later. Neither the antiserum nor the polysaccharide control gave an appreciable skin reaction.

Eighteen days after the first injection of egg albumin, all the animals were bled from the heart and killed. The autopsy findings will be discussed in a subsequent paper.

Experimental results. A. *The effect of compound E and ACTH on the Development of the Active Arthus State.* In Table I are recorded the size and appearance of the 24-hour skin lesions of each animal produced by the injections of egg albumin throughout the experimental period. The numbers presented in the columns are the product of the height,

FIG. 1, 2, 3—Twenty-four hour old skin lesions in rabbits produced by the 15th daily intracutaneous injection of egg albumin.

FIG. 1. Control rabbit No. 7 with edematous and hemorrhagic Arthus reaction.

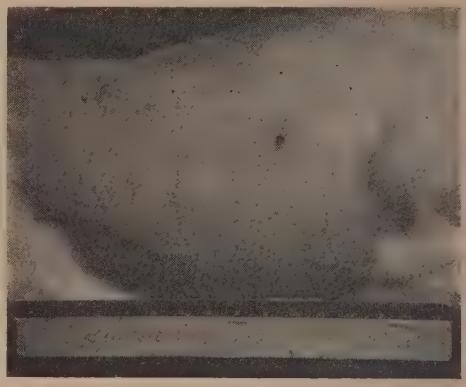

FIG. 2. Compound E-treated rabbit No. 13 with small, pale pink skin reaction. The black spot is made by gentian violet marking the site of injection. This lesion represents the largest ever obtained in the 8 Compound E-treated rabbits.

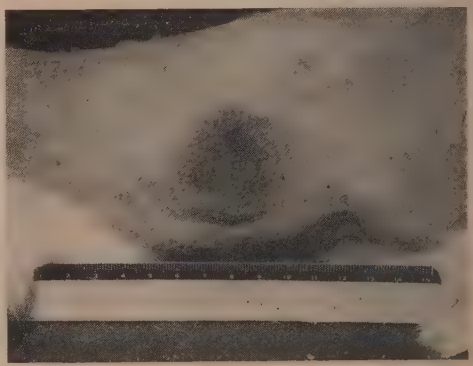

FIG. 3. ACTH-treated rabbit No. 23 with edematous and hemorrhagic Arthus reaction.

antibody. With the low titered sera there was close agreement. Thus if the standard precipitin method had been employed the differences observed between the low titered sera of most of the treated animals and the high titered sera of the controls would be even greater.

11. Fischel, E. E., and Kabat, E. A., *J. Immunol.*, 1947, v55, 337.

§ The height of the lesion was obtained by measuring the thickness of a doubled layer of skin at the site of greatest swelling, subtracting the thickness of a fold of normal skin and dividing by 2 to obtain the thickness of a single layer.

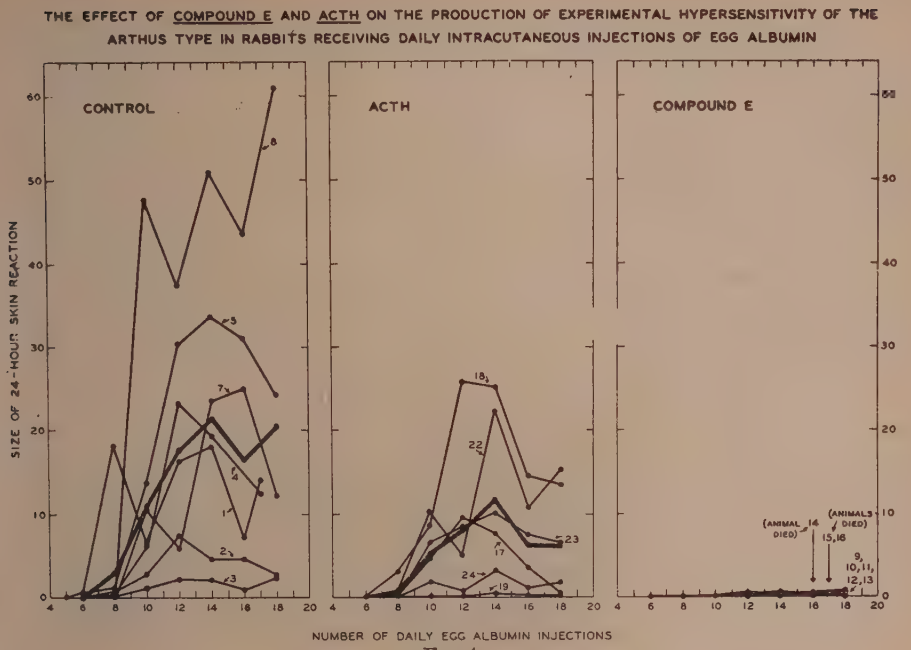

FIG. 4.

width, and length of these lesions.§ The results in Table I are graphically illustrated in Fig. 4 where the abscissa represent the number of the daily injections and the ordinate the magnitude of the lesion.

As shown in Table I and Fig. 1, 3 of the 8 control animals showed definite but small wheals on the 5th injection of egg albumin. On the 7th injection, wheals were produced in all of the 8 control animals and by the 13th injection, all of the 8 control animals had developed large Arthus reactions. In 6 of the 8 animals, in addition to extensive pink or red edema, the lesions contained large hemorrhagic and necrotic centers. In 2 of these animals (No. 5 and No. 8) the skin reactions were of such size that they occupied a large portion of the animal's side. These hemorrhagic and necrotic lesions often reached their greatest size after 48 hours, and healed with black scab formation.

In contrast, the 8 animals treated daily with 10 mg of compound E gave only slight or no skin reactions to egg albumin. The very slight reactions observed developed only after a much longer period of sensitization and consisted of flat, poorly defined pale pink areas in the skin. No extensive edema, hemorrhage or necrosis was seen.

Treatment with ACTH inhibited the production of the Arthus reaction but not nearly to such a marked degree as did treatment with compound E. As shown in Table I, 2 animals gave slight reactions, 2 animals gave moderate reactions and in only 3 out of the 7 were there hemorrhage and necrosis. One of the 8 ACTH animals (No. 20 not presented in the table) died on the 5th day.

B. *The effect of compound E and ACTH on antibody production.* Since, in the active Arthus reaction, antibody production is essential to the development of the reactive state, the possibility presented itself that the inhibition of reactivity by compound E and ACTH may have been due to the inability of animals under treatment to produce sufficient antibody.

In Table II are presented the anti-egg albumin antibody nitrogen content of 1 ml of serum from blood obtained from each animal

TABLE II.

Antibody Content of Sera from Rabbits Sensitized by Daily Intracutaneous Injections of Egg Albumin and Treated with ACTH or Compound E. (All sera were negative for anti-egg-albumin antibody on 0, 4 and 7 days).

Rabbit No.	Treatment	Antibody nitrogen content of serum, (μg/ml) on following days after first sensitizing injection of egg albumin				
		9	11	14	16	18
1	None, control	24	—	146	212	325
2	"	0	0	—	24	24
3	"	0	20	18	—	18
4	"	24	—	220	311	207
5	"	16	56	86	148	214
6	"	0	20	Dead		
7	"	8	24	84	180	200
8	"	8	44	90	236	304
Avg		10	27	107	185	185
9	Compound E	0	0	12	—	0
10	"	0	4	7	0	0
11	"	8	8	11	6	0
12	"	0	0	2	—	—
13	"	0	0	0	6	0
14	"	0	0	0	Dead	
15	"	0	0	3	0	Dead
16	"	0	4	0	0	Dead
Avg		1	2	4	2	0
17	ACTH	0	12	3	8	—
18	"	20	40	45	130	212
19	"	0	8	—	5	45
20	"	—	16	Killed		
22	"	8	24	46	76	82
23	"	20	28	64	118	130
24	"	0	8	8	6	7
Avg		8	19	33	57	95

at varying intervals as previously described. These results are presented graphically in Fig. 5. As is shown there, the sera of 5 of 7 control animals tested contained at least 200 μg of antibody nitrogen per ml at the termination of the experiment. The sera of the two other control animals contained 20 and 24 μg of antibody N per ml and these two animals gave the poorest Arthus reactions of the group (compare Fig. 4 and 5).

It is obvious from the table and figure that treatment with ACTH decreased the ability of the rabbits to produce antibody. Considering only the sera obtained from the final bleeding, ACTH suppressed antibody formation by an average of 50%. Only one of the sera of the 7 animals tested ever contained more than 200 micrograms of antibody N per ml. Again the 3 poorest skin reactors (Rabbits 17, 19, 24) had produced the smallest amount of antibody.

The effect of compound E on antibody production was more marked than that obtained with ACTH. None of the sera from the 8 animals receiving compound E ever contained more than 12 μg of antibody N per ml. At the end of the experiment, none of the sera of the 4 animals tested contained any detectable antibody by the technic employed.

As a confirmation of the precipitin reaction, the ability of the sera obtained by cardiac puncture at the termination of the experiment to produce passive anaphylaxis in guinea pigs was determined as shown in Table III. All of the sera tested from the control and ACTH-treated animals were able to passively sensitize guinea pigs to a shocking injection of egg albumin antigen. With serum from rabbit No. 6, 0.25 ml or less was sufficient to produce fatal sensitization. However, even 2.0 ml of serum from the compound E treated animals failed to sensitize the guinea pigs to even the slightest degree. It should be pointed out that as little as 32 μg of anti-egg albumin antibody nitrogen are required to produce fatal sensitization in 100% of the guinea pigs

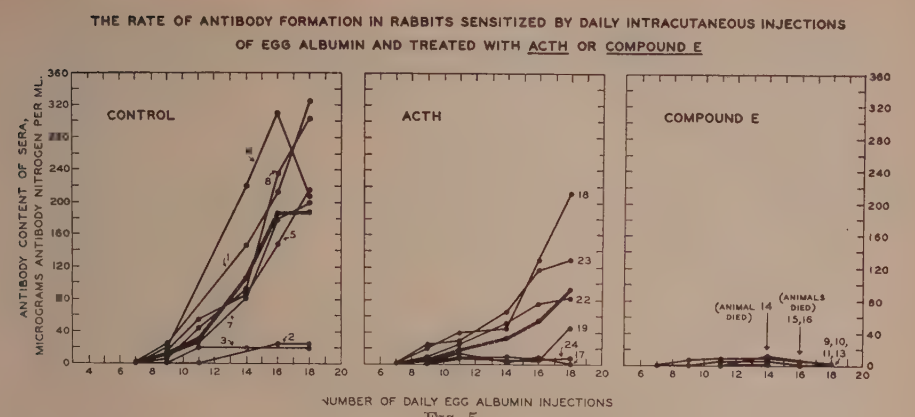

FIG. 5.

tested in this manner(12).

C. *The effect of compound E and ACTH on the passive Arthus reaction.* Although the suppression of antibody formation by treatment with compound E or ACTH could in itself fully explain the inhibitory effect of these two substances on the production of the allergic state, there was a further possibility that the animal's skin reactivity may have been altered by treatment. All of the animals were therefore tested for their ability to exhibit a passive Arthus reaction, when given antibody. Each rabbit was injected intracutaneously at two different skin sites with 1 mg and 0.5 mg respectively of rabbit antipneumococcus II antibody nitrogen contained in 0.4 ml of serum. Thirty minutes later, 1 mg of pneumococcus II polysaccharide was injected into each site. In columns 1 and 2 of Table IV are recorded the reactions observed 8 hours after the injection of the antigen. As is shown there, all of the rabbits both treated and controls, regardless of their ability to react actively to injections of egg albumin, produced a similar response to the intracutaneously injected antibody and antigen.

Discussion. The experimental data indicate that compound E and to a lesser extent, ACTH, suppress the development of the allergic state ordinarily produced in the rabbit by repeated intracutaneous injections of crystalline egg albumin. While 6 out of 8 control animals demonstrated large hemorrhagic and necrotic skin reactions following a preliminary period of sensitization, only 3 out of 7 of the ACTH-treated animals and none of the 8 rabbits receiving compound E produced lesions of similar severity. In all 3 groups of animals, there was a close correlation between the antibody nitrogen content of the sera and the severity of the skin reaction. The data show that the inhibitory effect of compound E and ACTH on the development of the Arthus state results from the ability of these hormones to suppress antibody formation, a prerequisite for the production of the active Arthus reaction(13).

Of particular interest is the fact that treatment with compound E and ACTH did not alter the animal's ability to produce the Arthus reaction when antibody was injected intracutaneously. During the passive Arthus reaction, all of the animals reacted in a similar manner and to the same degree regardless of treatment. This is particularly pertinent in view of the present emphasis on the general increased resistance to stress supposedly produced by treatment with these substances.

The mechanism by which ACTH and compound E suppress antibody formation is not apparent. The marked lymphoid atrophy and gluconeogenesis observed in these animals, which will be reported later, may in some way be related to the lack of antibody production.

12. Kabat, E. A., and Landow, H., *J. Immunol.*, 1942, v44, 69.

13. Culbertson, J. T., *J. Immunol.*, 1935, v29, 29.

TABLE III.

Relative Ability of Sera Obtained from Rabbits Sensitized by Repeated Intracutaneous Injections of Crystalline Egg Albumin and Treated with Compound E or ACTH to Passively Sensitized Guinea Pigs.

No. of rabbit from which serum was obtained	Treatment received	Volume of serum injected into guinea pig, ml	Reaction of guinea pig to 1 mg of egg albumin injected 48 hr later
1	None, control	.5	Dead, 5 min.
2	"	1.0	" 2 "
3	"	1.0	Moderate reaction
4	"	.5	Dead, 3 min.
5	"	.5	" 3 "
7	"	.5	" 3 "
8	"	.5	" 2 "
	"	.25	" 2 "
	"	.25	" 2 "
9	Compound E	2.0	None
10	"	2.0	"
11	"	2.0	"
13	"	2.0	"
18	ACTH	1.0	Dead, 2 min.
19	"	2.0	" 2 "
22	"	.5	" 3 "
23	"	.5	" 1 "
24	"	1.0	Moderate reaction

The lymphocyte has been suggested as the site of antibody formation. It is conceivable gluconeogenesis might decrease antibody formation by promoting the breakdown of the amino acids necessary for antibody protein synthesis.

Whether the therapeutic effects of compound E and ACTH in the rheumatic diseases are due to the suppression of antibody formation is a particularly interesting possibility in view of the evidence of an allergic mechanism in this group of diseases. Mirick(14), however, has reported that in man antibody to pneumococcal polysaccharides was produced as promptly and in as high titer in patients receiving ACTH or cortisone as in controls.

In the present experiment, ACTH suppressed antibody formation and the allergic state to a much less degree than did compound E. Since the effects of ACTH are produced through the adrenal cortex, the question is therefore raised as to whether under the conditions of the present experiment, the rabbits were able to produce large quantities of compound E-like substances in response to ACTH stimulation.

The presence of diarrhea, possibly of an infectious nature, in a large number of the compound E-treated animals should be noted. Diarrhea was observed in 5 of the 8 compound E-treated animals but only in 1 each of the 7 ACTH-treated animals and the 8 controls. The question may be raised as to whether the presence of diarrhea in the compound E-treated animals might account for their failure to respond to antigenic stimulation. However, diarrhea appeared in these rabbits after the controls had reacted to a marked degree to the egg albumin. Furthermore, 3 animals without diarrhea behaved in all respects similar to those in which diarrhea occurred. If the suppression of antibody response to crystalline egg albumin by compound E represents a specific case of a generalized phenomenon, the apparent increased susceptibility of compound E treated animals to diarrheal infection might be explained as failure to develop protective antibodies. In this regard, Antopol(15) has reported spontaneous

14. Mirick, G. S., Abstracts, 42nd Ann. Meeting, Am. Soc. Clin. Invest., 1950, 46.

TABLE IV.

Failure of Compound E and ACTH to Inhibit Passive Arthus Reaction in Rabbits. (To be contrasted to their striking effect on development of the Active Arthus Reaction).

Rabbit No.	Treatment	Size of skin wheal, cm, with	
		1 mg antibody N	0.5 mg antibody N
1	None, control	2.2 × 2.2 × 0.2 p.pk.	2.7 × 2.3 × 0.25 p.pk.
2	,,	3.2 × 2.2 × 0.2 pk.	3.0 × 3.2 × 0.3 pk.
3	,,	3.1 × 2.8 × 0.3 p.pk.	4.0 × 2.5 × 0.3 p.pk.
4	,,	2.4 × 2.2 × 0.3 p.pk.	3.5 × 2.1 × 0.25 p.pk.
5	,,	3.1 × 1.2 × 0.3 p.pk.	3.9 × 2.5 × 0.3 p.pk.
7	,,	3.5 × 2.8 × 0.25 pk.	4.0 × 2.7 × 0.3 pk.
8	,,	2.7 × 2.3 × 0.2 p.	2.7 × 2.8 × 0.25 p.pk.
9	Compound E	3.8 × 3.0 × 0.35 pk.	3.3 × 2.5 × 0.25 pk.
10	,,	2.5 × 2.5 × 0.3 pk.	2.6 × 2.1 × 0.25 pk.
11	,,	2.7 × 2.4 × 0.25 p.pk.	3.1 × 2.6 × 0.2 p.pk.
12	,,	2.5 × 2.0 × 0.15 p.pk.	2.0 × 3.1 × 0.15 p.pk.
13	,,	2.8 × 2.6 × 0.3 pk.	2.5 × 2.5 × 0.35 pk.
17	ACTH	3.3 × 2.3 × 0.35 pk.	2.5 × 1.7 × 0.25 pk.
18	,,	3.1 × 2.2 × 0.25 pk.	3.0 × 2.2 × 0.3 pk.
19	,,	2.8 × 1.7 × 0.3 p.	3.0 × 2.1 × 0.25 p.
22	,,	2.3 × 2.0 × 0.2 p.	3.3 × 2.4 × 0.25 p.
23	,,	2.1 × 2.1 × 0.25 pk.	2.9 × 2.7 × 0.2 pk.
24	,,	2.4 × 2.3 × 0.2 pk.	2.9 × 2.1 × 0.25 pk.

p = Pale. pk. = Pink.

The rabbits which had been receiving daily injections of egg albumin were passively sensitized at 2 different skin sites by the intracutaneous injection of 1 mg and 0.5 mg of rabbit anti-pneumococcus II antibody nitrogen, each contained in 0.4 ml of serum. Thirty minutes later, 1 mg of pneumococcus II polysaccharide was injected into each site. The 8-hour readings are recorded above. Neither the serum nor the polysaccharide control produced a skin reaction.

Corynebacterium pseudotuberculosis murium infection in mice under treatment with compound E while untreated controls remained uninfected.

Summary. 1. The effects of compound E and ACTH on experimental hypersensitivity of the Arthus type and on antibody production in the rabbit are described. Both compound E and ACTH inhibited sensitization to repeated intracutaneous injections of crystalline egg albumin. Moreover the inhibition produced by compound E was much greater than that obtained with ACTH; with compound E sensitization was almost completely prevented in all of the 8 animals tested.

2. The data indicate that the inhibitory effect of compound E and ACTH on the development of the Arthus state results from the ability of these hormones to suppress antibody formation. As shown by antibody nitrogen determinations, compound E and ACTH suppressed antibody formation by an average of 100 and 50% respectively.

3. In contrast to their striking effect on the production of the active Arthus reaction, compound E and ACTH had no effect on the passive local Arthus reaction, when antibody is supplied to the animal. Thus, the capacity of the animal to react to antibody-antigen combination in the tissues is not altered by treatment.

15. Antopol, W., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 262.

Received July 5, 1950. P.S.E.B.M., 1950, v74.

Development of Chloramphenicol-Resistant and Chloramphenicol-Dependent Variants of a Strain of *Klebsiella pneumoniae*.* (18059)

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During the course of attempts to enhance the resistance of several pathogenic bacteria to some of the new antibiotics a variant of one of the strains was encountered which failed to grow in the absence of chloramphenicol. This paper deals chiefly with the details of the emergence and isolation of this "chloramphenicol-dependent" strain.

Source of the strain. The organism used at the beginning of this study was a strain of *Klebsiella pneumoniae*, type A, which was originally isolated from the urine of a patient with pyelonephritis and meningitis. As far as could be determined, the patient had not previously received any antibiotic therapy. At the time when the study was begun, the strain had been subcultured only a few times on the surface of agar plates and grew profusely in large mucoid colonies.

Materials. Brain heart infusion broth (Difco) pH 7.4, was the liquid medium used for suspending or growing the organisms and for making serial dilutions of cultures or antibiotic. The solid medium for surface growth was heart-infusion agar (Difco), pH 7.4. Crystalline chloramphenicol (Chloromycetin) was supplied by Dr. E. A. Sharp of Parke, Davis & Co. Initial solutions of the drug were made in distilled water and all subsequent dilutions were made in broth prior to their incorporation in proper amounts in the media to be used.

Methods. A series of agar plates containing 2-fold dilutions of chloramphenicol was freshly prepared each time before use. Plain agar plates without antibiotic were prepared at the same time. The inoculum for subcultures was prepared by suspending a 5 mm loopful of a surface growth in 0.4 ml of broth; a 2 mm loopful of this suspension was then streaked on a sector of each of a number of agar plates containing various concentrations of the antibiotic in an appropriate range and also on a plate containing no antibiotic. The plates were incubated at 37°C for 48 hours and then inspected for growth with the naked eye and with the aid of an engraver's glass (3x). The growth was recorded as follows: O = no discernible growth; +++ = full growth, comparable to that of a control culture not previously exposed to drug; ++ and + = intermediate growth, and ± = pin-point colonies seen with the aid of the lens. If only a few colonies developed, their number was recorded. In the attempt to enhance the resistance of the strain, the inoculum for each successive subculture was prepared from the plate containing the highest concentration of chloramphenicol on which there was moderate but incomplete growth (+ or ++).

Results. The minimum concentrations of chloramphenicol which completely inhibited growth and the minimum concentrations which produced partial inhibition (++ or less) in 30 successive subcultures made in this manner are shown graphically in Fig. 1. In the lower portion of this figure there is also indicated the degree of growth which occurred when an inoculum from the same suspension was streaked on the surface of antibiotic-free agar at the time of each of the successive transfers from the agar which contained the antibiotic.

There was a more or less progressive increase in the resistance of the strain to chloramphenicol in successive subcultures between the sixth and twenty-fourth transfers. At the time of the first 6 exposures of the original strain to chloramphenicol its growth was completely inhibited by 3.1 μg per ml and partially inhibited by 1.6 μg per ml of agar, with some variations that were within the limits of error of the method. Beginning

* Aided by a grant from the United States Public Health Service.

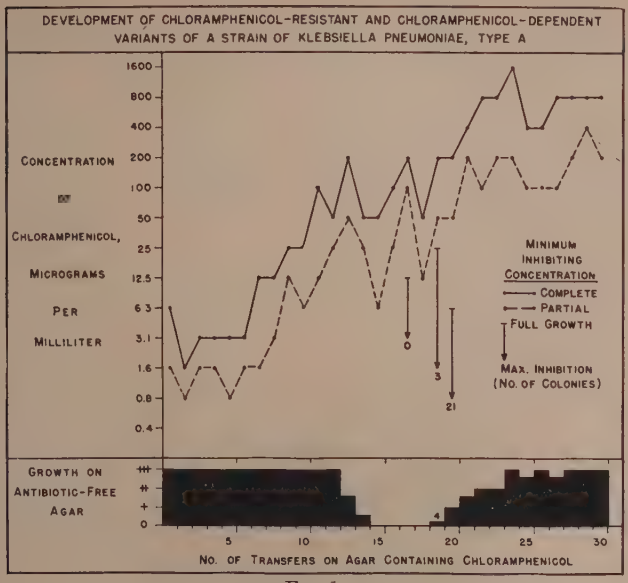

FIG. 1.

with the twenty-second transfer the strain was completely inhibited in most instances by 800 μg/ml and partially inhibited by 200 μg/ml.

On the antibiotic-free agar plates, the growth of the successive subcultures from the chloramphenicol agar plates appeared maximal for the first 12 transfers. The 13th and 14th transfers from drug-containing to drug-free agar showed progressively diminishing growth and the 15th through the 18th of these transfers yielded no discernible growth on the antibiotic-free agar. The 19th similar transfer yielded only 4 colonies on drug-free agar and subsequent subcultures from the chloramphenicol-agar plates produced progressively increasing growth on the antibiotic-free agar to the 24th transfer; after that the growth on antibiotic-free agar was at or near maximum.

Growth was maximum or nearly so throughout the early transfers to the plates which contained chloramphenicol in lower concentration than those which are indicated in Fig. 1 as showing partial inhibition. Up to the 17th transfer, however, only 2 or 3 dilutions were included beyond the first one which permitted maximal growth. Subsequent transfers included lower dilutions down to concentrations of 1.6 or 0.4 μg of chloramphenicol per ml. In each of 3 of these transfers, namely the 17th, 19th and 20th, there was a progressive decrease in the amount of growth in decreasing concentrations of chloramphenicol until little or no growth occurred. This is indicated in Fig. 1 by the arrows which begin at the level of the least concentration that yielded maximum growth and point at the greatest concentration in which maximum inhibition of growth again occurred.

These findings suggested that the suspension of organisms made from the 14th to the 17th subcultures on chloramphenicol-agar consisted largely, if not wholly, of organisms which required chloramphenicol for growth. In other words, these organisms were "chloramphenicol-dependent." It was also evident that (1) a minimum of 3.1 μg or more of chloramphenicol per ml of agar was usually required to permit any appreciable amount of growth of these dependent organisms, (2) optimum growth occurred on agar containing 12.5 or 25 μg per ml, (3) higher concentrations of the antibiotic again inhibited growth and (4) it was nevertheless possible to continue to

TABLE I.
Some Additional Transfers of the Chloramphenicol-Dependent Culture.

Conc. of chloramphenicol in agar, μg/ml	Growth of subcultures				
	Series A Regular series, 17th transfer*	Series B Growth from Plate *a* of series A	Series C Growth from Plate *b* of series A	Series D Growth from Plate *c* of series C	Series E Growth from Plate *d* of series D
400	0	0	0	0	0
200	0	0	±	0	±
100	+a	0	±	0	±
50	+++	0	+++c	0	+++
25	+++	±	+++	±	+++e
12.5	+++	++	+++	++	+++
6.3	±	++	++	++d	++
3.1	0	±	±	±	±
1.6	±b	0	±	0	0
0.8	0	0	0	0	1
None	0	0	0	0	1

+++ = full growth; ++, + ± = diminishing growth, and numerals indicate number of colonies where growth was minimal.

* From agar plate containing chloramphenicol, 25μg/ml.

The growth from "e" was used for subcultures in broth (Table II).

enhance the resistance of the strain by additional subcultures in progressively higher concentrations of antibiotic, using as the inoculum for each subculture organisms which had survived exposure to the highest concentration that was partially inhibitory.

A number of separate subcultures of the chloramphenicol-dependent organisms were also made from plates containing various concentrations of chloramphenicol to further series of plates containing serial dilutions of the antibiotic. The results of some of these subcultures are shown in Table I. Within rather wide limits, the concentration of the drug to which the chloramphenicol-dependent organisms were last exposed did not greatly affect the range of concentrations which would support their growth on the next subculture.

Attempts to quantitate the growth of the chloramphenicol-dependent organisms were somewhat hampered by difficulties in obtaining good growth on subculture from agar plates to broth containing the antibiotic. The growth on the plate which contained 25 μg of chloramphenicol per ml in Series E (designated as "e" in Table I) was suspended in broth and aliquots of this suspension were inoculated into tubes of broth containing the antibiotic in concentrations of 6.25, 12.5, 25 and 50 μg per ml, respectively. Almost no visible growth resulted at first but serial subcultures were nevertheless made at 48 hour intervals from each of these tubes to others containing the same amount of drug. The fifth subculture showed moderate growth in the broth containing 6.25 and 12.5 μg per ml, barely visible growth in the one containing 25 μg and no discernible growth in the tube containing 50 μg of antibiotic per ml of broth.

The fifth subculture in 12.5 μg of chloramphenicol per ml of broth was chosen for further quantitative studies. Serial 100-fold dilutions of this culture were made and 2 series of agar plates were poured with 1 ml of each dilution of organisms; one set of plates contained 25 μg of chloramphenicol per ml and a parallel series contained no antibiotic. The number of colonies that developed in each of these plates is shown in Table II. These results suggested that all but a small proportion of the organisms originally present in the broth culture were chloramphenicol-dependent and that probably all of the organisms represented in the 14 colonies which developed from the 10^{-6} dilution of this culture in agar containing 25 μg of antibiotic per ml were chloramphenicol-dependent.

A single colony from the latter plate (designated as "f" in Table II) was then transferred to broth containing 12.5 μg of chloram-

TABLE II.
Growth of Chloramphenicol-Dependent Strain After 5 Subcultures in Broth Containing 12.5 μg of Chloramphenicol per ml.

Dilution of broth culture	No. of colonies per ml: In agar containing chloramphenicol, 25 μg/ml	No. of colonies per ml: In antibiotic-free agar
10^{-2}	+++	4
10^{-4}	145	1
10^{-6}	14f	0
10^{-8}	0	0

f—One of these colonies was used for further subcultures (Table III).

TABLE III.
Growth of Chloramphenicol-Dependent Strain in Agar Containing Various Amounts of the Antibiotic.

Conc. of chloramphenicol, μg per ml	No. of colonies*
400	0
200	0
100	3 g
50	1426 h,i,j,k
25	560 l,m,n,o
12.5	235
6.3	19
3.1	1
1.6	16
0.8	15 p
0.4	20 q
0	0
0	3r

* From 1 ml of a 10^{-4} dilution of a 72-hour culture (in broth containing 12.5 μg of chloromycetin per ml) of one of the colonies (f) indicated in Table II. These plates were read after 72 hr incubation. The letters signify isolated colonies which were subcultured, as indicated in Table IV.

phenicol per ml. Moderate growth occurred after incubation for 72 hours. This growth was then diluted 1:10,000, and 1 ml of this dilution of culture was incorporated into each of a series of agar plates containing graded concentrations of chloramphenicol. The plates were incubated for 72 hours and the number of colonies which developed in each of the plates is shown in Table III. A few colonies developed in plates containing only small amounts of chloramphenicol and a total of 3 colonies developed in 2 plates of antibiotic-free agar. This suggested that a few organisms had now emerged which were no longer chloramphenicol-dependent.

Finally, 12 individual colonies, indicated by separate letters in Table III, were picked from some of the plates. Each colony was emulsified in 0.2 ml of broth and the suspensions were used to inoculate on the surface of a new series of agar plates containing graded concentrations of chloramphenicol. The growths obtained after 48 hours are indicated in Table IV. The colonies which had developed in the antibiotic-free agar and those which were picked from the agar containing less than 1.6 μg of chloramphenicol per ml yielded growth of organisms which were essentially as sensitive to the antibiotic as a culture of *K. pneumoniae* that had not previously been exposed to the drug. They also grew profusely on antibiotic-free agar. On the other hand, the colonies that were picked from the plates containing 25, 50 and 100 μg per ml all yielded organisms that were chloramphenicol-dependent. These failed to grow in the absence of chloramphenicol or on concentrations lower than 3.1 μg per ml. Furthermore all of the latter cultures grew best on the agar which contained 25 μg of chloramphenicol per ml.

Detailed comparisons of the sensitive, resistant and dependent variants were not made but a number of observations were recorded in the course of this study. The colonies of the dependent organisms which developed on optimum concentrations of chloramphenicol were large and mucoid and resembled those which had never been exposed to the antibiotic. The colonies of the resistant variant that grew in the later transfers on both chloramphenicol-agar and on antibiotic-free agar were smooth, smaller and no longer mucoid in character. All of the variants obtained from plates on which good growth had occurred retained their microscopic morphology and staining characteristics. The chloramphenicol-resistant and chloramphenicol-dependent variants, however, had lost their serological specificity with respect to the Quellung reaction in homologous antiserum, but the same was also true of the control organisms which had been maintained by serial transfer on antibiotic-free agar.

Discussion. A number of workers, notably Miller and Bohnoff(1,2) and Paine and Fin-

TABLE IV.
Growth of Organisms from Individual Colonies Derived from a Chloramphenicol-Dependent Strain.

Colony*	Last exposure†	Concentration of chloramphenicol, μg/ml. 400	200	100	50	25	12.5	6.3	3.1	1.6	0.8	0.4	0
g	100	0	0	±	+++	+++	±	0	0	0	0		0
h	50	0	0	0	±	±	±	0	±	0	0		0
i	50	0	0	0	+	++	++	±	0	0	0		0
j	50	0	0	0	++	++	+++	±	±	0	0		0
k	50	0	0	0	++	++	+	+	0	0	0		0
l	25	0	0	0	++	+++	++	+	±	0	0		0
m	25	0	0	0	++	+++	++	+	±	0	0		0
n	25	0	0	±	++	+++	++	±	0	0	0		0
o	25	0	0	0	++	+++	++	±	0	0	0		0
p	.8	0	0	0	0	0	0	±	+	++	+++	+++	+++
q	.4	0	0	0	0	0	0	±	+	++	+++	+++	+++
r	0	0	0	0	0	±	±	+	++	++	+++	+++	+++
Control‡	0	0	0	0	0	0	0	0	++	+++	+++	+++	+++

* See Table III.
† Chloramphenicol, μg/ml.
‡ Strain of *K. pneumoniae* not previously exposed to chloramphenicol.

land(3,4) have succeeded in developing streptomycin-resistant and streptomycin-dependent variants from originally sensitive strains of a number of different organisms after exposing them to this antibiotic. This subject has been reviewed recently by Miller and Bohnhoff(5). As far as could be ascertained, organisms which are dependent for growth on any of the newer antibiotics have not previously been described.

The course of events leading to the emergence and isolation of chloramphenicol-resistant and chloramphenicol-dependent variants of a strain of *Klebsiella pneumoniae* that was originally sensitive to this antibiotic have been detailed in this paper. As was the case with the streptomycin-dependent variants previously developed in this laboratory(4) and with those described by Miller and Bohnhoff(2), there was a critical concentration of antibiotic in which the chloramphenicol-dependent variant grew most vigorously. As the concentration was increased above this level or decreased below it, the growth of the chloramphenicol-dependent variant decreased progressively until it completely failed to grow. It is of interest that the chloramphenicol-dependent variant emerged during the step-grade increase in the resistance of the strain by successive exposures to increasing concentrations of the antibiotic and it was first recognized when the resistance of the strain to chloramphenicol had attained this critical level. As variants of greater resistance appeared in later transfers in the chloramphenicol-containing medium, they gradually submerged the dependent variants.

In point of fact, it was largely a matter of chance that the dependent variant was recognized at all. Its recognition was due to the fact that the particular inoculum used for some of the subcultures consisted entirely of chloramphenicol-dependent variants so that little or no growth occurred on the antibiotic-free medium. Had there been in these inocula a sufficient proportion of chloramphenicol-resistant variants which were also capable of growing in the absence of the antibiotic, the presence of the dependent variants could readily have gone unrecognized by the method used in this study.

1. Miller, C. P., and Bohnhoff, M., *Science*, 1947, v105, 620.

2. Miller, C. P., and Bohnhoff, M., *J. Bact.*, 1947, v54, 467.

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The strain used to initiate the present study was one of 14, each of a different species, which were employed in attempts to enhance the resistance of bacteria to 4 new antibiotics other than penicillin and streptomycin. In no other instance was a dependent variant recognized although each strain had been transferred 40 times in media containing graded concentrations of the respective antibiotics and from the antibiotic-containing media also to antibiotic-free media. It cannot be stated with any assurance that such dependent variants had not developed in other strains or to other antibiotics since the method used was not suitable for their recognition unless they replaced the sensitive and resistant variants completely, or nearly so, at some stage in the course of the subcultures.

No attempts have been made to study the genetic pattern of the emergence of the resistant and dependent variants and of the "back mutations" to more sensitive and more resistant variants. The possible significance of these variants with respect to the mechanism of action of antibiotics has been discussed previously in relation to the streptomycin-dependent variants(4).

Summary and conclusions. The resistance of a strain of *Klebsiella pneumoniae* to chloramphenicol was enhanced 128-fold by serial subculture on the surface of agar plates containing graded concentrations of this antibiotic.

A chloramphenicol-dependent variant also emerged during the course of these subcultures. This variant grew best in the presence of a critical concentration of chloramphenicol; as the concentration of antibiotic was either increased progressively beyond this critical level or decreased below it there was a steady decline in growth until complete inhibition of growth occurred.

The chloramphenicol-dependent variant was first recognized soon after the resistance of the strain had been enhanced to a point where it was completely inhibited by this critical concentration.

Evidence was also obtained of possible "back-mutation" to variants which were as sensitive as the parent strain.

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Hemorrhagic Skin Lesions Produced by Intradermal Meningococcus Toxin in Rabbits following Treatment with ACTH or Cortisone.*(18060)

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Inhibition of the Shwartzman phenomenon by ACTH has been described by Soffer, Shwartzman, Schneierson, and Gabrilove(1). These authors reported that the phenomenon was prevented in 8 out of 10 rabbits when 12.5 mg of ACTH was injected intramuscularly 2 hours prior to the intravenous or "provocative" injection of meningococcus toxin. Comparable amounts of ACTH had no inhibitory effect when given on the preceding day, before the intradermal or "preparatory" injection of toxin.

In preliminary experiments, a similar result was obtained in this laboratory. When ACTH was administered to 8 rabbits in 2 separate doses of 10 mg Armour Standard ACTH, 6 and 4 hours before the intravenous injection of toxin, the gross intradermal hemorrhage which characterizes the Shwartzman phenomenon failed to occur in every case, while typical reactions were produced in each of 4 control rabbits. However, it was noted that the skin sites prepared with

* This work was supported by research grants from the National Multiple Sclerosis Society and the Life Insurance Medical Research Fund.

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TABLE I.
Occurrence of Hemorrhagic Skin Reactions in Rabbits Following Intradermal Injection of Meningococcus Toxin and Intramuscular Injection of ACTH or Cortisone.

Exp. No.	No. of rabbits	Drug	Time of injection (hr) Before prep. 24	Before prep. 4	After preparation 0	4	8	12	18	20	24	28	Results: Hr after preparation	Results: Lesions†
1	2	ACTH		10*	10	10	10						18	2/2
2	3	”							10	10			24	3/3
3	8	”			10			10					16	8/8
4	2	”			10	10	10		10	10			18	2/2
5	3	”				10			10				18	3/3
6	2	Cortisone		30	30	30	30						30	2/2
7	2	”	10	10		10							20	2/2
8	4	”				10	15			15			30	4/4
9	2	”							10	10	10	10	48	2/2
10	26	Untreated controls											36	0/26

* Numbers refer to dosage, in mg, of Armour Standard ACTH or of Cortisone, at the indicated hour.

† Numerator shows number of rabbits with reaction of pallor and petechiae at prepared skin site. Denominator shows total number in group.

meningococcus toxin became extremely pale within a few hours after ACTH treatment, and many small petechiae appeared in these areas. In order to determine whether the lesions were the result of partial inhibition of the Shwartzman phenomenon, or were brought about by ACTH, rabbits were given intradermal injections of meningococcus toxin and treated with ACTH or Cortisone, without being given the customary intravenous injection of toxin. The present report is concerned with the skin lesions which were observed following such treatment.

Method. Intradermal injections of 0.5 cc of meningococcus “agar washings” filtrates, prepared by the method of Shwartzman(2), were made in the shaven abdominal skin of male adult hybrid rabbits weighing approximately 2 kg. Two strains of Group I and II meningococci were used as sources of toxin, and an additional sample of standard toxin (44-B) was obtained through the kindness of Dr. Gregory Shwartzman. In most experiments, 2 dilutions (1:1 and 1:4) of 2 different toxins were injected in separate areas on each side of the abdomen. The toxins were shown to be effective in the usual Shwartzman reaction before being tested with ACTH and Cortisone; the results observed with different toxins in the experiments to be described were qualitatively similar.

Two lots of ACTH† were employed with activity corresponding to 20% and 30%, respectively, of Armour Standard ACTH. The dosages employed are expressed below in terms of Standard ACTH. In all experiments, the material was injected subcutaneously or intramuscularly in the thigh, with 10 mg suspended in 5 cc sterile saline.

Cortisone (11-dehydro-17-hydroxycorticosterone-21-acetate, Merck) was suspended in sterile saline in a concentration of 10 mg in 2 cc, and injected intramuscularly in the thigh.

Effect of ACTH on skin areas prepared with meningococcus toxin. Eighteen rabbits were prepared by intradermal injections of meningococcus toxin and injected with ACTH at varying intervals of time, as shown in Table I. In 2 animals, 4 injections of 10 mg each were given at 4-hourly intervals, beginning 4 hours before skin preparation. In the remainder of the group, total amounts ranging from 20 to 50 mg were given in divided doses at the time of preparation and thereafter.

Twenty-six control rabbits received intra-

† Supplied through the cooperation of Armour and Co., Chemical Research and Development Department, Chicago, Ill.

dermal injections of meningococcus toxin and were observed during the next 36 hours without further treatment.

Results. In all of the 26 control animals, the skin areas injected with toxin became reddened and slightly edematous after approximately 4 hours, and after 24 hours the lesions in every instance consisted of pink, slightly elevated, indurated swellings measuring 3-4 cm in diameter (Fig. 1-A). No hemorrhages or dilated vessels were grossly visible in the lesions.

In contrast, the rabbits receiving ACTH exhibited the following changes in the skin areas prepared with toxin: When ACTH was given before or at the time of the intradermal injection, edema and erythema were slight or failed to occur at all. When ACTH was started after edema had already appeared, the edema subsided within the next few hours. In 15 rabbits (Table I, Exp. 1, 3, 4, and 5) approximately 8 hours after the injection of toxin, oval or circular areas of pallor were seen at the injected skin sites, corresponding in size to the erythematous zones in control rabbits. At this time, small dilated blood vessels formed a rim around the periphery of each area of pallor. After 16-18 hours, the central portions exhibited dark blue discolorations caused by a superficial meshwork of engorged small blood vessels, and scattered petechiae appeared at the periphery (Fig. 1-B). In all 18 rabbits, between 18 and 30 hours after skin preparation, petechiae of varying sizes appeared throughout the injected areas and in some instances became confluent, resulting in grossly hemorrhagic lesions (Fig. 1-C). The lesions differed from the typical Shwartzman reaction in that they were flat and possessed irregular margins, and the spots of hemorrhage were usually distributed unevenly through the involved area, in contrast with the diffusely swollen, uniformly purple appearance of the Shwartzman phenomenon. Moreover, the development of the lesions was gradual, often continuing over a period of 36 hours with increasing degrees of vascular engorgement and ecchymosis, in contrast with the abrupt and rapid development of hemorrhage in the Shwartzman reaction.

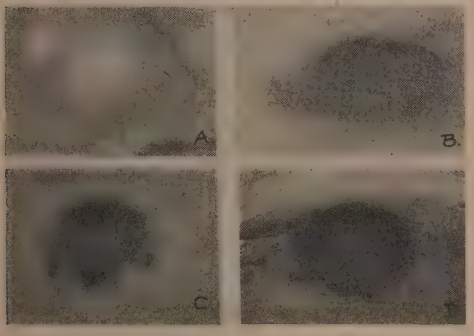

FIG. 1.
Reactions in rabbit skin 24 hr after intradermal injection of meningococcus toxin.
(A) Control. (B) After 20 mg ACTH. (C) After 30 mg ACTH. (D) After 30 mg Cortisone.

The optimal dosage of ACTH for the production of lesions was not accurately determined, because of limitation of the supply of material. In 8 rabbits which received 2 mg each, before and after skin preparation, no lesions occurred. With 40 mg of ACTH the lesions were more deeply hemorrhagic than with 20 mg. When ACTH was given at or before the time of skin preparation and followed by 2 or 3 additional doses at 4 hour intervals, the eventual lesions were more extensive than when ACTH administration was started on the following day.

Effect of Cortisone on prepared skin. Ten rabbits were given intramuscular injections of Cortisone, in total doses ranging from 30 to 120 mg each (Table I). In 4 animals, Cortisone was given both before and after the intradermal injection of toxin; in 6 it was given only after the toxin.

Results. In all 10 animals, pallor and vascular engorgement followed by the appearance of petechiae were observed in the prepared skin sites. An illustrative lesion is shown in Fig. 1-D. The reactions were similar to those described above in ACTH-treated rabbits, but confluent hemorrhages were less frequently observed. A longer time was required for the development of lesions following Cortisone. In 8 rabbits petechiae were not observed until 30 hours or longer after skin preparation. In 2 animals which were given Cortisone 24 hours prior to skin injection, lesions developed in the prepared skin after 20 hours.

Comment. In untreated rabbits, hemorrhage does not occur in skin sites prepared with meningococcus toxin until after an intravenous injection of toxin or other suitable "provocative" material is given, 18 or more hours later. A variety of apparently unrelated substances are capable of producing the phenomenon by intravenous injection, including bacterial toxins, whole bacteria, starch, agar, glycogen, kaolin, and antigen-antibody mixtures(2,3). The possibility cannot be excluded that ACTH and Cortisone produced the observed reactions by virtue of a comparable "non-specific" property which is not related to the known functions of these materials. It is unlikely that bacterial contaminants in the substances were responsible for the lesions, since cultures of the ACTH and Cortisone preparations yielded no growth.

It is possible that the skin reactions in ACTH- or Cortisone-treated rabbits may be based on a mechanism different from the Shwartzman phenomenon itself. In the absence of edema and erythema, and in the gradual progressive manner of their development, the lesions differed sharply from the typical Shwartzman reaction. Conceivably, the abatement of the usual inflammatory reaction to locally injected toxin may have increased the vulnerability of skin tissue to a primary damaging property of the toxin. There is evidence which suggests that the vessels of skin become less permeable following an injection of meningococcus toxin(4), and the possibility that further interference with permeability may occur following the administration of ACTH and Cortisone is under investigation.

Summary. The observations indicate that under certain circumstances the treatment of rabbits with ACTH or Cortisone results in a type of local skin damage by meningococcus toxin which is not seen in untreated animals. The application of these findings to other varieties of tissue damage by bacteria and their products is a field which merits further study.

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A Method for Visualization of Kidney Blood Vessels Applied to Studies of the Crush Syndrome.* (18061)

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A variety of conditions such as crush syndrome, severe trauma to muscles, non-traumatic muscular ischemia, transfusions with incompatible blood, heat stroke, toxemias of pregnancy, sulfonamide intoxications, poisoning of different types, etc., have one clinical manifestation in common, oliguria or anuria. Furthermore, the pathological finding of lower nephron nephrosis seems to be typical for all the above mentioned conditions. A drop in blood pressure below the necessary filtration pressure would itself be expected to cause anuria as filtration in the glomeruli stops. Arterial blood pressure determinations in the conditions mentioned will usually, in the very early stages, show a lowered blood pressure, but not always a pressure below that believed adequate for ultrafiltration. Often an increase in blood pressure is seen as the condition develops; despite this the patient may be completely anuric.

Three main theories are generally accepted as being possible explanations of oliguria and

* This investigation was supported by funds from the Dr. Henry C. Buswell Memorial.

anuria: (a) renal ischemia, (b) tubular obstruction, and (c) unselective reabsorption of glomerular filtrates.

The first hypothesis (a), which claims that renal ischemia is the main factor leading to lower nephron nephrosis, has recently gained strength by the important investigations of Trueta and co-workers(1), at least as a complication of the crush syndrome in rabbits. The technic used in those experiments has been further perfected by Barclay(2).

Baker and Dodds(3) have supported the theory (b) that the mechanism of oliguria is caused by an obstruction of the tubules. Others have the same point of view(4,5). Most investigators, however, oppose this hypothesis(6-14).

The theory (c) of unselective reabsorption of glomerular filtrates was first put forward by Dunn and his co-workers(15,16), and later confirmed by Richards and his associates(17).

Inasmuch as the hypothesis (a) of renal ischemia has gained a growing acceptance as the cause of these conditions, it seemed important to study the kidney circulation in normal rabbits and rabbits which had been exposed to crush injury, using Trueta's method of producing crush. These studies appeared to be especially indicated inasmuch as there was available a method for intra vitam visualization of the walls of the blood vessels.

Method. The method used in these experiments has been described in a previous publication by Schlegel(18). The principle of the method is to inject intravenously a fluorescent dye, Thioflavine-S (methyldehydrothio-p-toluidine-sulfonate.† This dye has the property of being taken up by the walls of the blood vessels immediately after the injection, thus making these visible when observed in ultraviolet light. Initially the tissues were examined in ultraviolet light after freezing and dehydration, but recently we have introduced a modification which seems to be of value. Immediately after removal of the kidney, it is sliced with a sharp knife, and the slices are placed in 100% glycerin. This will clear the tissue so that the vascular details are clearly visible with the microscope when observed in reflected ultraviolet light. The diffusion of the fluorescent dye out into the glycerin is slow enough to allow observations for hours, and photographic recordings have routinely been carried out.

The dye does not cause any change in blood pressure and the investigations of Algire and Schlegel(19) have shown that the dye causes no change in the state of blood vessels when observed by means of transparent windows in mice. This dye when injected intravenously into the living animal will give a picture of all vessels through which blood was flowing at the time of injection. Whatever happens during the removal of the organ to be studied

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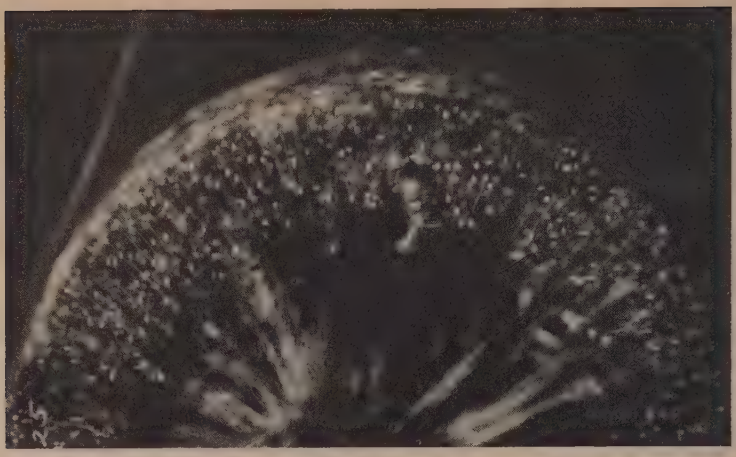

FIG. 1.
Photomicrograph taken in incident ultraviolet light of a kidney from a rabbit with a blood pressure of around 40 mm mercury. Note brilliant fluorescence of all vessels and practically no diffusion of dye (8×).

FIG. 2.
Photomicrograph taken in incident ultraviolet light from a normal rabbit kidney. Kidney was removed 15 sec. after injection of Thioflavine-S. Medulla shows a brilliant fluorescence (8×).

does not interfere with the interpretation of the results, since the dye is already in the walls of the vessels and will remain there for some time before it diffuses out (Fig. 1). No other fluorescent dyes we have tried have the property of making the blood vessels

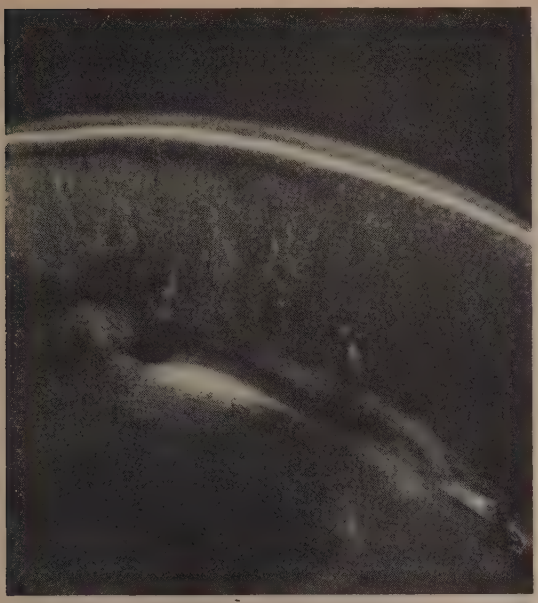

FIG. 3.
Photomicrograph taken in incident ultraviolet light of a kidney from a crush rabbit. Kidney was removed 15 sec. after injection of Thioflavine-S. Note lack of fluorescence of medulla as compared to Fig. 2 (8×).

visible because they diffuse out of the blood vessels almost simultaneously with the intravenous injection, whereas diffusion of Thioflavine-S takes several minutes, thus, allowing adequate time for removal of the organ to be studied. The blood, in itself, shows no fluorescence, so that squeezing of blood from the vessels will not interfere with the results. The rate of diffusion of the dye out of the blood vessels into the tissue is probably determined by the blood pressure, the rate of blood flow, and the permeability of the vessels. These conditions are to be investigated further. Blood pressure determinations have been carried out on most of the rabbits by inserting a needle in one of the carotid arteries, and recordings have been made on a kymograph by means of a mercury manometer. To determine the urinary excretion of the rabbit, collection of urine by catheterization has also been carried out in most of the experiments, usually in a half-hour period immediately prior to the injection of Thioflavine-S and removal of the kidneys.

Material. For these preliminary experiments, 46 rabbits have been used. Dial‡ in the amount of 0.6 cc per kg, given intravenously has been used for anesthesia.

Thioflavine-S in a 4% aqueous solution has been injected in an ear vein in the amount of 1 cc per kg of body weight. At different time intervals after the injection, the kidneys have been removed, sliced, placed in glycerin, and examined with the microscope in reflected ultraviolet light.

A tourniquet was applied to the left thighs of 16 rabbits according to the method described by Trueta, and left for 4½ hours. These rabbits will hereafter be referred to as "crush rabbits." In these animals the dye has been injected 1 to 2 hours after removal of the tourniquet.

Results. In all the kidneys examined, there has not been a single case in which the inter-

‡ Dial (each cc contains Diallylbarbituric acid 0.1 g, urethane 0.4 g, monoethylurea 0.4 g, distilled water *q.s.*) Furnished through courtesy of Ciba Pharmaceutical Products, Inc., Summit, N.J.

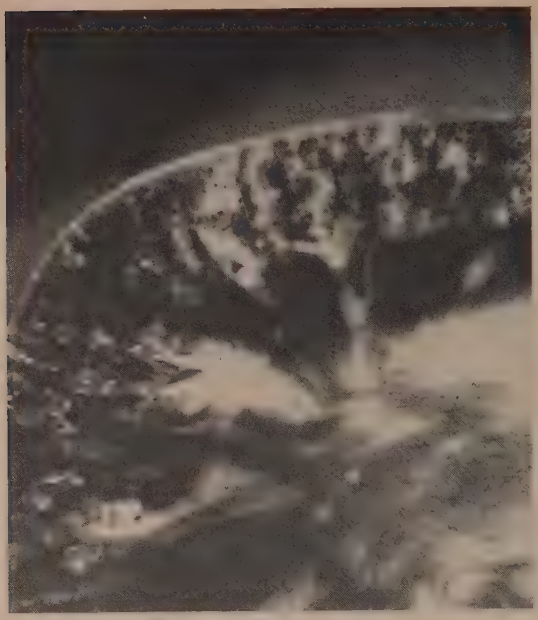

FIG. 4.
Photomicrograph taken in incident ultraviolet light of normal rabbit kidney after intravenous adrenalin injection (8×). Note that fluorescence is not limited to any particular area.

lobular arterioles supplying the glomeruli did not show definite fluorescence. The interlobular arterioles and all the glomeruli, the peripheral as well as the juxta-medullary, showed a high degree of fluorescence in the kidneys of normal as well as of crush rabbits. Fig. 2 and 3 are photographs of sections from kidneys removed 15 seconds after the injection of Thioflavine-S. Fig. 3 is the kidney of a rabbit which had been exposed to a crush injury according to Trueta's method, and Fig. 2 is from a kidney of a normal rabbit. From the photographs it can be seen that the glomeruli, in both instances, show a bright fluorescence, indicating that blood had been flowing through the glomeruli in the normal rabbit kidney and in the kidney from the crush rabbit, causing the same degree of fluorescence of the glomeruli. A difference exists in the degree of fluorescence of the medulla. There is a bright fluorescence of the medulla in the kidneys from the normal rabbits contrasting with only very slight fluorescence of the medulla in the kidneys from the crush rabbits. The urine output in the rabbits exposed to the crush injury within 1-2 hours after removal of the tourniquet was on the average much higher than that of the normal rabbits. Phenolsulfonphthalein kidney function tests showed a delayed and decreased excretion of the dye as compared to the excretion in normal rabbits.

Blood pressure determinations were carried out in most of the experiments and showed on the average a pressure of around 100 mm mercury in the normal anesthetized rabbits and around 70 mm mercury in the crush rabbits.

Discussion and conclusion. We have not been able to confirm the results of Trueta's experiments in rabbits exposed to a crush injury. He interprets his results as indicating the exclusion of the renal cortex from the circulation and a maintenance of a blood flow through the medulla. The results we have obtained seemed rather to indicate a decrease in blood flow in the medulla of the crush rabbits; a decrease which is seen at a time

FIG. 5.

Photomicrograph taken in incident ultraviolet light of a crush rabbit after intravenous injection of adrenalin in a dosage equivalent to that given the rabbit whose kidney is seen in Fig. 4. Note that there is no significant difference from Fig. 4.

when the blood pressure is usually around 70 mm mercury. There does not seem to be any decrease in the blood flow through the glomeruli, as their fluorescence is practically equivalent to the fluorescence of the glomeruli of a normal rabbit kidney. The increased output of urine 1-2 hours after removal of the tourniquet indicates that the glomeruli are functioning. Whether the increase in urine production is caused by an increase in ultrafiltration or by a decrease in tubular reabsorption of water cannot be answered yet; the delayed and diminished output of phenolsulfonphthalein is most likely caused by a decreased tubular function.

In order to show whether adrenalin would cause any distribution of the blood flow as indicated by Trueta in the crush, adrenalin in the amount of 3.7 cc per kg (1:10,000) was given intravenously to a normal rabbit (Fig. 4) and to one which had been exposed to crush injury (Fig. 5). It will be seen from these pictures that there is an obvious vasoconstriction with a diminution of the blood flow; moreover the picture seen does not show any indication of a juxta-medullary by-pass. We must therefore conclude that in our experiments we have not confirmed Trueta's results indicating a by-pass in case of a crush injury. Our experiments seem to indicate that the changes in kidney function in rabbits seen 1-2 hours after a crush injury are a result of a decrease in blood flow, particularly in the medulla, caused by a drop in systemic blood pressure, most likely combined with a constriction of the efferent arterioles.

Summary. The technic for visualization of blood vessels by intravenous injection of the fluorescent dye, Thioflavine-S, has been used in a study of kidney circulation in normal rabbits and rabbits exposed to crush injury.

The results obtained show that the blood flow in the medulla is decreased. We have not been able to confirm Trueta's results indicating a by-pass through the juxtamedullary glomeruli.

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Production of Acute Pancreatitis with Ethionine and its Prevention by Methionine.*† (18062)

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It has been repeatedly shown(1-4) that a low protein high fat diet produces, in experimental animals, morphological changes in the pancreas, in addition to fatty infiltration of the liver. In one study, methionine reversed these changes(4). In addition, malnutrition in young children in tropical zones results in a fatty liver and fibrotic changes in the pancreas, as observed in South Africa(5), Central Africa (Kwasiorkor)(6,7), and the Dutch West Indies(8). Recently, it was reported from Hungary(4) that infants on deficient diets revealed a decreased pancreatic secretion, relieved by the administration of milk. The relationship of a deficiency of animal protein to those syndromes in children has been discussed(9).

In previous studies(10,11), it was observed that ethionine, (α-amino-γ-ethylthiolbutyric acid), a presumed antagonist of methionine, inhibits the incorporation of radioactive methionine and glycine into the proteins of some tissues *in vivo* (protein synthesis) and induces a rapid fatty infiltration of the liver, without concomitant necrosis. The fatty liver occurs in adult female but not in adult male rats(12). A similar sex difference was also found in the inhibition of protein synthesis in the liver(13). All these effects of ethionine were prevented by the administration of methionine. During the course of these studies, pancreatic lesions were observed.

Experiments and results. Rats, previously fasted for a period of 12 to 16 hours, were injected intraperitoneally with ethionine, (1 mg per g body weight) in 3 divided doses. Within 24 hours the pancreas became enlarged, prominent and yellowish white. Histologically, the basal and perinuclear basophilia had almost completely disappeared; the eosinophilic cytoplasm was swollen and crowded with fine zymogen granules. The normally seen pyroninophilic material of the acinar cells was also strikingly diminished. At the base of a few cells, vacuoles were seen which failed to stain with fat stains (Fig. 1B). This lesion was entirely prevented by the simultaneous administration of methionine, but not by glucose (which also inhibits the fatty liver) nor by cysteine. It appears in equal severity in males and females, despite the absence of the fatty liver in the former (Table I). Forty-eight hours after the administration of ethionine, the acinar structure was obscured by spotty coagulation necrosis of the cytoplasm of some of the acinar cells and marked vacuolization of others. Only a few of the vacuoles gave a

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†We are obliged for technical assistance to Mr. Morey Slodki.

‡ Fellow in Cancer Research of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

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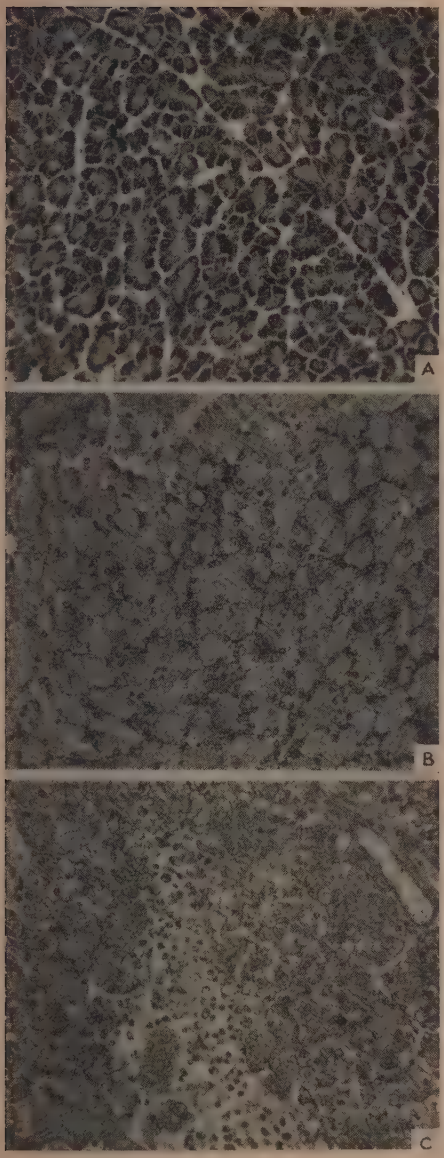

FIG. 1.

Photomicrographs of rat pancreas (×200).

A. Control (after 24 hours fasting). B. 24 hours after ethionine administration. Loss of basal basophilia of acinar cells, which are swollen and contain many zymogen granules. C. 48 hours after ethionine administration. Necrosis of acinar cells, loss of acinar structure and interstitial inflammatory infiltration.

staining reaction for fat. The severe cytoplasmic alterations were not accompanied by conspicuous nuclear changes except for occasional chromatolysis. They were associated with focal infiltration of the edematous interstitial tissue with histiocytic cells intermixed with a few neutrophilic segmented leucocytes (Fig. 1C). These lesions were more marked and frequently associated with fat necrosis in rats sacrificed after longer intervals. The islets and ductal cells revealed no characteristic alterations.

Discussion. It is safe to assume that the acute pancreatitis produced by ethionine is due to some interference with the normal utilization of methionine. Analogy with the other organs(10), invites the suggestion that inhibition of protein synthesis (due to methionine deficiency) causes the lesion in an organ which is known to be very active in protein synthesis. The observed loss of basophilia of the acinar cells, early in the process, is of interest, in view of the recently emphasized relation between cytoplasmatic basophilia (associated with ribonucleic acids) and protein metabolism(14). This aspect deserves further investigation. The rapidly developing pancreatitis, as a result of interference with methionine (and possibly protein) metabolism tends to confirm the opinion that the pancreatic lesion due to malnutrition in the tropics is caused by protein deficiency. The presented findings may also suggest that episodes of disturbed protein metabolism cause the initial stage of the commonly encountered chronic relapsing and of the acute pancreatitis in the temperate zone. It should be stressed that the pancreatic lesion, though often occurring simultaneously with the fatty liver, can develop independently of it and need not respond to the same treatment as the liver does.

Summary. The administration of ethionine produces in rats loss of cytoplasmic basophilia of the pancreatic acinar cells, followed by diffuse pancreatitis. The lesion is prevented by methionine but not by cysteine or glucose which latter prevents fatty liver following

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TABLE I.
Effect of Various Compounds Upon Pancreatic and Hepatic Changes Produced by Ethionine.

No. animals	Sex	Duration of exp., hr	Administered		Pancreas		Total liver lipids* % (wet wt) ± Standard dev.
			Ethionine*	Additional	Loss of basophilia	Necrosis	
3	f	24	—	—	0	0	5.1 ± 0.5
3	m	24	—	—	0	0	5.2 ± 0.7
3	f	24	+	—	+	0	14.6 ± 0.1
3	m	24	+	—	+	0	5.0 ± 1.2
3	f	24	+	Methionine‡	0	0	4.9 ± 0.1
3	f	24	+	Cysteine‡	+	0	14.4 ± 2.3
6	f	24	+	Glucose§	+	0	5.3 ± 0.3
3	f	24	+	Choline chloride‖	+	0	13.5 ± 2.5
6	f & m	48	—	—	0	0	5.2 ± 0.2
6	f	48	+	—	+	+	18.2 ± 5.8
3	f	48	+	Methionine‡	0	0	4.8 ± 0.3

* 1 mg per g body weight intraperitoneally.
† Determined as described previously(11).
‡ Administered intraperitoneally in amounts equimolar to and simultaneously with ethionine. In addition, one-half molar quantities were administered every 12 hr until time of sacrifice.
§ 2.5 g by stomach tube, in 3 divided doses, simultaneously with ethionine and 1.5 g 12 hr later.
‖ 42 mg intraperitoneally in 3 divided doses, simultaneously with ethionine and 10.8 mg 12 hr later.

ethionine administration. The lesion is interpreted as a result of interference with methionine (and possibly protein) metabolism.

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Plasminogen Purification by Acid Extraction.* (18063)

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Several methods have been reported for the partial purification of the active form of serum protease (see Tagnon *et al.*(1) and Rocha e Silva and Rimington(2) for references). Relatively few attempts have been made to isolate the naturally-occurring inactive form (plasminogen, profibrinolysin, serum tryptogen, etc.) in a state of high purity. The preparations commonly used have consisted essentially of the euglobulin fraction of serum or plasma, obtained by salting out, dialysis, or dilution and acidification (Milstone(3), Christensen(4,5), Christensen and MacLeod(6), Kaplan(7), Holmberg(8), MacFarlane and Pilling(9), Ratnoff (10).) In these preparations the enzyme

* This study was supported in part by grants from the Life Insurance Medical Research Fund and the Masonic Foundation for Medical Research and Human Welfare.

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represents only a small portion of the total proteins, and in addition, such preparations are contaminated by variable amounts of inhibitor and antistreptokinase. Oncley and associates(11) obtained subfractions of human plasma containing plasminogen and prothrombin by ethanol fractionation. However, their method of fractionation involved spontaneous activation of a portion of the plasminogen in the presence of fibrin and other proteins, resulting in contamination of the preparation by active plasmin and by the split products of fibrin. Recently Cohn and associates(12) have reported a newer method of plasma fractionation, but information regarding purity and yield of the plasminogen-containing fraction has not been published. The most highly purified preparations described up to the present are those of Remmert and Cohen(13), who reported purification of 135-165 fold as compared with whole serum. Other procedures have been published, Loomis, George and Ryder(14); Richert (15), but insufficient data on original and final purity have been given to evaluate the amount of purification obtained. Remmert and Cohen experienced difficulty with their procedures because of the inconsistent behavior of different preparations during purification.

The present paper details a simple procedure involving a hitherto undescribed property of plasminogen, solubility in dilute mineral acids, by means of which purification of as much as 250 fold or more may be obtained.

Materials and methods. 1. Streptokinase prepared by modification of methods described previously (Christensen, 1950) was obtained from Lederle Laboratories.† The preparation contained about 550 streptokinase units per microgram of nitrogen. For use as activator in fibrinolytic assays, it was diluted to contain 3000 units per assay tube, since preliminary titration showed this to be the optimum quantity to give maximum plasminogen activation under the conditions of this assay. In determination of casein proteolysis by the method of Remmert and Cohen(1), aliquots of the plasminogen preparations were activated with 10-fold dilutions of streptokinase. In the majority of cases maximum activation was obtained with 150 to 1500 units of streptokinase in the digestion mixture.

2. Fibrin: Armour's Bovine Fibrinogen, clotted with Lederle Hemostatic Globulin† in the presence of plasminogen and streptokinase was used as substrate for fibrinolytic assays.

3. Casein: Several preparations, including one obtained from Dr. Philip Cohen, were employed. Results with different preparations were of the same order of magnitude, but not identical. In order that our results might be comparable with those of Remmert and Cohen, assays reported in the present paper were carried out with their sample of casein.

4. Several preparations containing plasminogen activity were available. Two lots of plasma fraction III,‡ prepared by Harvard method 9 (Oncley *et al.*)(4) were available in the lyophilized state. In addition a fraction of human placental extract†, a by-product of the commercial preparation of placental immune globulin, was available as a frozen paste and as a lyophilized powder.

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† We are indebted to Mr. Frank Ablondi of Lederle Laboratories for generous gifts of Hemostatic Globulin and placental fractions as well as preparation and purification of special lots of streptokinase for these studies.

‡ One lot of plasma fraction III was obtained through the kindness of Dr. Dwight Mulford, Division of Biologic Laboratories, Massachusetts State Department of Health. The second lot of plasma fraction III was obtained from the American National Red Cross through the courtesy of Dr. L. E. Strong.

TABLE I.
Comparison of Acid and Buffer Extraction of Plasminogen from Plasma Fraction III and Placental Fraction III.

Extracting medium	Total activity*	Total nitrogen	Purity (units/mg N)
	Plasma fraction III		
0.2 N H_2SO_4	2.0×10^6	37 mg	53,000
Saline phosphate buffer pH 7.5	2.5×10^6	280 mg	8,930
	Placental fraction III		
0.2 N H_2SO_4	0.8×10^6	265 mg	3,020
Saline phosphate buffer pH 7.5	0.8×10^6	750 mg	1,066

* Activity in fibrinolytic units.

TABLE II.
Purification of Acid Extracts by Precipitation and Reextraction with Acid.

Extract No.	Total activity*	Total nitrogen	Purity (units/mg N)
	Plasma fraction III		
1	2.88×10^6	85.56 mg	33,600
2	0.768×10^6	2.88 mg	266,000
	Placental fraction III		
1	0.7×10^6	116.5 mg	6,000
2	0.3×10^6	7.0 mg	42,900
3	0.18×10^6	2.76 mg	65,200

* Activity in fibrinolytic units.

This material, on the basis of its method of preparation, is probably equivalent to plasma fraction III.

5. Fibrinolytic protease assays were performed by the method described previously (Christensen, 1950). One unit of plasmin is that amount, in a digest mixture of 1.0 ml, which will lyse the standard fibrin clot in 30 minutes at 35°C. For greater accuracy, the lysis times of serial dilutions of the protease are plotted, and the dilution lysing in 30 minutes obtained by interpolation on the curve.

6. Proteolysis of casein was determined as described by Remmert and Cohen(1), using a sample of casein prepared by them.

Experimental. Preliminary experiments with various procedures for protein fractionation, as well as with the procedures described by Remmert and Cohen(1) confirmed their view that the precipitation behavior of plasminogen preparations is inconsistent, and that there appears to be a marked tendency for plasminogen to co-precipitate with other proteins. It was decided, therefore, to attempt selective extraction rather than precipitation of the enzyme. A variety of extraction procedures, including extraction at varying pH levels with dilute solutions of alcohols and salts, were tried without notable success. Dilute solutions of mineral acids, however, were found to extract all plasminogen activity from the starting materials. The procedure is based on extraction of the starting material with H_2SO_4 or HCl for a period of 15-30 minutes at room temperature. The normality of the acid is not critical, the usual concentration employed being 0.2N H_2SO_4. However, satisfactory results have been obtained with concentrations between 0.1N and 0.05N. The ratio of dry weight of crude plasminogen to acid does not appear to be critical. Satisfactory preparations have been obtained with concentrations (dry weight) of crude plasminogen of 4-10% in the extracting medium. The usual practice has been to extract 200-500 g of frozen placental extract (about 20-50 g dry weight) in 500 ml 0.2N acid, or 10 g dried plasma fraction III in 200 ml acid. Following acid extraction, the insoluble debris is removed in an angle centrifuge. The acid supernatant is brought to 0.05M phos-

phate and the pH adjusted to about 7.5 with NaOH. If desired, the sulfate ions may be removed before neutralization by treatment of the acid extract with sufficient Amberlite IR-4-B (Rohm and Haas Company) to raise the pH to about 7. Phosphate is added as above and the pH adjusted with acid or base. A slight, finely-divided precipitate usually forms on neutralization. If this precipitate is removed, a portion of the activity is lost. The purity of the first acid extract is variable, even from aliquots of the same lot of crude plasminogen. The cause of this variability has not been ascertained. The minimum purity obtained has been of the order of 3000 fibrinolytic units per mg. In general, the preparations from plasma fraction III average about 50,000 units per mg of nitrogen, while preparations from placental fraction III are less pure. In Table I are presented the results of typical extractions of placental and plasma fractions with acid and with saline phosphate buffer at pH 7.5.

The crude plasminogen preparations tested have been characterized by the presence of variable amounts of lipoid material in the extracts, greater in the case of the placental preparations than in the plasma fractions. The lipoidal material can be removed by a preliminary extraction of the crude preparations with alcohol or acetone, followed by ether.

The initial acid extracts may be further purified by the following procedure. The acid or neutralized extract is adjusted to pH 5.2 with alkali or acetic acid. The precipitate which forms is removed by centrifugation and reextracted with 0.2N H_2SO_4. If necessary this precipitation at pH 5.2 and reextraction with acid may be repeated. Table II illustrates typical results obtained by this procedure with placental fraction III and plasma fraction III.

Plasminogen preparations obtained in the above manner show little development of spontaneous activity, at most a fraction of 1% of the total.

The sulfuric acid extracts are less stable than the neutralized extracts. Stability was determined by storing acid and neutralized extracts of placental and plasma fractions in the refrigerator at 3°C and in a water bath at 35°C. At intervals, aliquots were removed and the residual fibrinolytic activity determined. The results of this experiment are presented in Fig. 1.

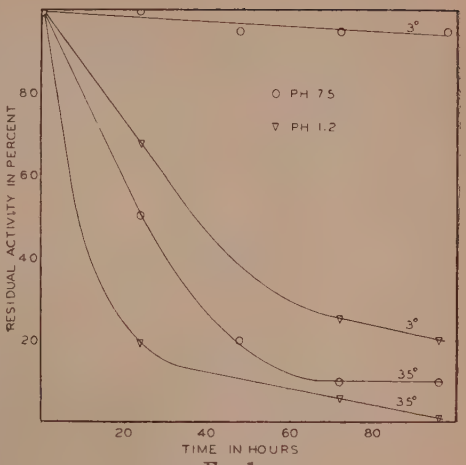

FIG. 1.

Decrease in activity of acid-extracted plasminogen at 3°C and 35°C. One sample (pH 1.2) is the original acid extract. The second sample (pH 7.5) was adjusted with NaOH after adding phosphate to 0.05 M.

As may be seen from the data in Fig. 1, the acid extracts at pH 1.2 are quite unstable at both 35° and 3°C, practically all activity being lost in 4 days. On the other hand, the neutralized extract at 3°C showed only slight loss of activity during this period. However, at 35°C, the neutralized extract was also unstable. Spontaneous activity did not develop in these preparations as indicated by their inability to lyse fibrin clots in the absence of streptokinase.

Remmert and Cohen(13) have reported purification of about 165-fold over serum, representing a purity of 19 casein proteolytic units per mg nitrogen. Analysis of plasminogen preparations, purified by acid extraction, by the casein proteolysis method indicates that purities of 20-30 casein units per mg nitrogen are frequently obtained, indicating a purification of more than 250-fold, a figure which is in agreement with the purification factor calculated from fibrinolytic data.

Summary. The procedures described above

utilize a previously undescribed property of plasminogen, solubility in dilute mineral acid, to extract it from placental and plasma fractions. Purification of over 250-fold has been obtained by successive extractions with 0.2N H_2SO_4 and precipitations at pH 5.2. The total yield has ranged from a minimum of about 10% to almost complete recovery of the enzyme present in the starting material, with an average recovery of about 30%.

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Observations on Pain and Temperature Perception within the Sternal Marrow Cavity. (18064)

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It is generally known that during the performance of a sternal marrow aspiration, the subject usually experiences an aching pain of one to five dol† intensity at the moment of actual aspiration of marrow contents. This pain is separate and distinct from any discomfort that the patient may experience during the introduction of the aspiration needle and it is not abolished by procainization of the skin and periosteum. In most areas of the body specifically studied, both pain and temperature can be perceived. Many workers (1,2) have shown that distension of the stomach with sufficient force results in pain. Wolf and Wolff(2) have demonstrated that a sense of warmth or cold is experienced when either water warmer than 40°C or colder than 18°C is circulated within the stomach. It is common experience that either pain or temperature sensation may be elicited by suitable stimulation of the skin. The normal bladder possesses both pain and temperature sensation(3).

Therefore, we wished to determine the presence or absence of temperature perception within the sternal cavity.

Sternal marrow aspiration was performed in 3 healthy, young adult males. The procedure was preceded by anesthetization with 4 cc of 2% procaine of the skin, deep tissue and periosteum overlying the site of the puncture. Great care was taken to ensure complete anesthesia of these structures before introducing the needle. An 18 gauge needle was employed. The gross appearance and the stained smears of the material aspirated gave proof that the needle was in the marrow cavity. In each subject warm or cold isotonic saline was injected in random order using a 2 ml capacity Luer glass syringe. Care was taken that the subject received no cue as to the temperature of the saline either from comments of the examiners or from spillage on the skin.

During the entire procedure, the first subject noted, in addition to the pain incident to withdrawal of the marrow sample, a deep burning pain of one to 2 dols intensity. Following marrow aspiration, while the needle remained in the cavity, 4 injections of 1 ml each of isotonic saline were given. The temperature of the 2 injections of cold saline solution was approximately 18°C and of the

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† Hardy, Wolff and Goodell(4,5) define the "dol" as a unit of painfulness equal to 2 just noticeable differences in pain intensity of pain. Ten dols equal the maximum pain.

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warm 40-45°C. Twenty to 30 seconds were required to complete each injection. At the beginning of each injection there was an aching pain which was in every way similar to that experienced during aspiration. This pain diminished before the injection had been completed. There was no temperature sensation experienced by the subject with either the hot or the cold saline solution.

The second and third subjects did not experience the constant deep burning pain noted by the first, but they did experience pain during the initial seconds of saline injection. Neither of these subjects experienced any temperature sensation with the injection of 1.5 ml of isotonic saline ranging in temperature from 16°C to 45°C. No sensations of pressure were felt by the subjects during any of the procedures.

We conclude from these observations that structures in the sternal marrow cavity are pain sensitive while being insensitive to temperature within the range studied. The pain results from traction and pressure upon these sensitive structures without giving rise to a sense of pressure or fullness.

Summary. Pain and temperature sensitivity of the structures in the sternal bone marrow cavity was tested on three normal subjects. Four injections of 1 ml of hot (40-45°C) and cold (16-22°C) saline were made through the anesthetized skin and periosteum of each subject. No temperature sensations were reported although a deep burning pain and an aching pain were reported at the beginning of each injection.

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Metabolites of Pamaquine in Urine.* (18065)

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Pamaquine, (8-(4-Diethylamino-l-methylbutylamino)-6-methoxyquinoline), a derivative of 8-aminoquinoline, exerts a curative action in mosquito-transmitted vivax malaria presumably by attacking a tissue form of the plasmodium which is responsible for relapses (1-4). Two pieces of evidence suggest that the compound exerts its activity *in vivo* through a metabolic transformation product. One, there is no correlation between the mean plasma concentration of pamaquine and its antimalarial effect(3) and two, the drug, although it exerts considerable activity against plasmodia *in vivo*, is relatively inactive in growing cultures of erythrocytic parasites(5).

The usefulness of pamaquine is limited by the frequent occurrence of toxic effects with doses in the therapeutic range. The adverse reactions include the regular production of methemoglobin and the occasional occurrence of acute hemolytic anemia. Since the drug does not produce methemoglobin nor hemolysis *in vitro*, except in concentrations far in excess of those achieved in plasma

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

† Now at the National Heart Institute, National Institutes of Health, Bethesda, Md.

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on therapeutic dosage, it appears likely that its toxicity also is mediated through a metabolically derived product.

Although pamaquine is known to be extensively metabolized in the body(6) little is known concerning its pathway of metabolism. A recent paper by Hughes and Schmidt(7) indicates that pamaquine and its homologues are transformed, in part, in the rhesus monkey to water soluble derivatives with acidic properties. These substances were not identified. In the present work, a preliminary study has been made of certain of the basic metabolic products of pamaquine in urine and plasma. The specific purpose of these studies was to acquire information which bears on the possibility that such metabolic transformation products might account for the toxic or therapeutic effects of pamaquine.

Experimental. It was observed that a few drops of urine or plasma of humans or dogs who were receiving pamaquine produced methemoglobin when added to hemolysed red cells, and hemolysis when added to intact red cells. Erythrocytes of dogs were much more susceptible to the hemolytic action than were those of humans. Below pH 5-6, urine stored at low temperature retained these properties for considerable periods of time but lost them with progressively increasing rapidity as the pH was raised. The loss in oxidative ability almost exactly paralleled the loss in ability to lyse erythrocytes, suggesting that the two properties were possessed by the same substance or substances.

Partial purification of the material responsible for the effects on blood was accomplished by extracting it from cooled urine at pH 10 into cooled chloroform and then returning it to a pH 6 phosphate buffer. This procedure resulted in the loss of a considerable portion of the activity because of the instability of the material at the alkaline pH necessary for its extraction. The extracted material appeared yellow and showed an absorption maximum at 460 mu. However, this absorption may have been due to the presence of other metabolic transformation products. Attempts at further purification were unsuccessful because of the instability of the substance.

8-Aminoquinoline administered to dogs also imparted to the urine the two properties of hemolysing red cells and converting hemoglobin to methemoglobin. 8-Aminoquinoline induced these effects only *in vivo,* but 5-hydroxy-8-aminoquinoline was found to hemolyse red cells and to form methemoglobin *in vitro.*

The urine and plasma of patients and dogs receiving pamaquine contained another transformation product which was highly fluorescent in acid solution. This substance was found to be stable in acid and alkaline solution. It possessed neither methemoglobin-forming nor hemolytic properties. Unlike pamaquine it did not couple with diazotized sulfanilic acid. The substance was partly purified as follows: It was extracted from alkaline urine into ethylene dichloride, and then returned to 0.1N H_2SO_4. The acid solution was made alkaline and extracted several times with heptane. The basic metabolite in the heptane was then returned to 0.1N H_2SO_4. The acid phase was then adjusted to pH 6 and extracted several times with petroleum ether. This served to remove pamaquine, while leaving the metabolic substance in the aqueous phase. The absorption spectrum of the substance was measured and was found to be distinctly different from that of pamaquine (Fig. 1). The sharply defined peak of the absorption curve at 250 mu suggests the presence of a single absorbing substance, though it is possible that it is representative of a mixture of two or more products. No attempt was made to isolate the material in crystalline form because of the minute amounts of material involved.

The fluorescent metabolite in urine could be roughly estimated by the methyl orange reaction(8) on the assumption that its molecular weight is about the same as that of the parent drug. Subjects receiving 60 mg per day of pamaquine excreted 0.3-2 mg of the

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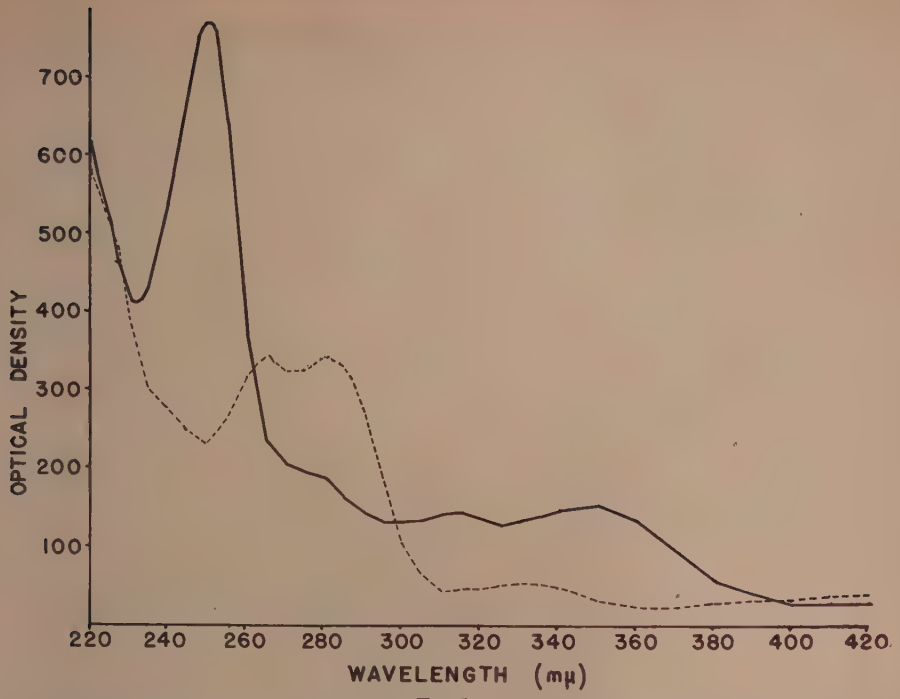

FIG. 1.
The absorption spectra of pamaquine (broken line) and the fluorescent metabolic product of pamaquine (solid line) in 0.1 N H_2SO_4. Concentration of pamaquine was 10 μg per ml; that of the metabolite was unknown. Cell thickness = 1 cm.

metabolite in the urine per day, and exhibited mean plasma levels of 5-30 μg per liter. These plasma levels are considerably lower than those of the parent compound (6). One subject was unusual in that he attained a plasma level of the substance of 350 μg per liter and excreted about 10 mg per day.

A homologue of pamaquine, 8-(3-diethylaminopropylamino) - 6 - methoxyquinoline, which differs only in the composition of the side chain, was administered to dogs and was also found to transmit to the urine a fluorescent substance and a substance or substances with hemolytic and methemoglobin-producing properties. Both these materials had solubility characteristics similar to the ones formed from pamaquine.

Discussion. Since neither of the two pamaquine metabolites was isolated in crystalline form, it was not possible to identify them. The fact that 8-aminoquinoline forms methemoglobin and lyses erythrocytes *in vivo,* but not *in vitro,* whereas its 5-hydroxy derivative is active *in vitro,* suggests that 8-aminoquinoline and its derivatives, such as pamaquine, may be converted in the body to 5-hydroxy derivatives, which then act as methemoglobin formers through reversible conversion to a quinonimine (Blanchard and Schmidt) (9).

It is possible that the oxidant metabolic material found in plasma and urine is directly responsible for at least some of the noxious effects of pamaquine in man. Its presence in blood suggests a direct link with the formation of methemoglobin. Its connection with the occurrence of acute hemolytic anemia is not clear since the latter occurs only seldom, whereas methemoglobinemia results uniformly. It may be that this substance causes hemolytic anemia when some other predisposing factor is also present.

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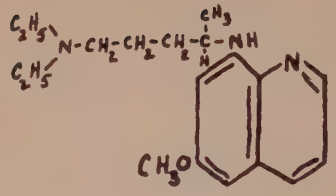
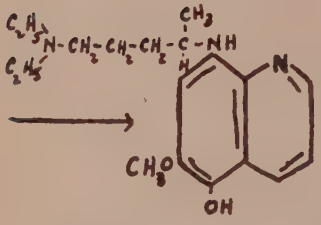
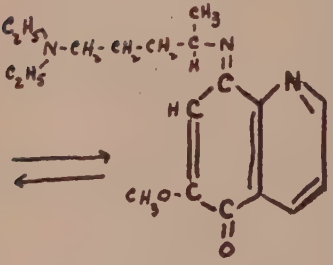


Pamaquine



5-Hydroxy Pamaquine



Quinonimine of Pamaquine

The possibility exists that both the toxicity and therapeutic effects of pamaquine are mediated through the same metabolic derivative. This is an important consideration in assaying the chances of obtaining an 8-aminoquinoline which is curative in malaria without being toxic. It is of interest that Greenberg, Taylor and Josephson have found that whereas pentaquine, (8-(Isopropylaminoamylamino)-6-methoxyquinoline) exerts a negligible effect on the erythrocytic form *P. gallinaceum in vitro*, its 5-hydroxy derivative exerts considerable activity on this parasite(10).

Summary. Two metabolites of pamaquine have been demonstrated in the plasma and urine of humans and dogs receiving pamaquine. One of these is an unstable compound which can, *in vitro*, convert hemoglobin to methemoglobin and lyse erythrocytes. The other is a stable compound which is highly fluorescent. Neither of these substances has been isolated in crystalline form.

It is possible that the substance with the oxidant and lytic properties is responsible for the toxic actions of the parent drug. The answer as to whether it also accounts for its therapeutic effect awaits definition of its antimalarial activity.

A homologue of pamaquine which differs only in its substitution on the side chain, yields metabolic products with properties similar to those obtained with pamaquine.

10. Greenberg, J., Taylor, D. J., and Josephson, E. S., personal communication.

Received July 12, 1950. P.S.E.B.M., 1950, v74.

Histochemical Alterations Revealed by Tetrazolium Chloride in Hypertensive Kidneys in Relation to Renal VEM Mechanisms.* (18066)

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Tetrazolium salts provide a new tool for the study of metabolic activity of living cells (1-3). The compound used in the present

* Supported by grants from the New York Heart Association, the Josiah Macy, Jr., Foundation, Eli Lilly and Company, the National Institute of Health, United States Public Health Service, and the Postley Hypertension Fund, and by a grant from the Leukemia Research Foundation, Inc., to one of us (M. M. B.).

study, 2,3,5-triphenyl tetrazolium chloride (TTC), is a colorless, water soluble substance which yields on reduction a deep red, water insoluble formazan. The intracellular reduction of this compound results from its interaction as a hydrogen acceptor with tissue dehydrogenases, the insoluble formazan being deposited locally in the cells in patterns which vary in different tissues. There is as well an excellent correlation between the oxygen consumption of tissue as measured by conventional Warburg technic and the extent of deposition of formazan. The formazan can be extracted from the cells by solvents such as acetone and measured quantitatively in a photoelectric colorimeter.

The present study is concerned with the histochemical appearance of kidneys of normal and hypertensive animals and human subjects after treatment of the tissues with TTC. Our findings indicate that a correlation exists between the microscopic pattern and the alterations in kidney VEM metabolism which occur in human and experimental renal hypertension. In view of the potential value of this method as an indication of the participation of a renal factor in different hypertensive syndromes, these studies have been extended to other hypertensive states, including that produced by DCA, and will be reported elsewhere.

In the normal kidney the renal vasoexcitor, VEM, is produced consistently by the cortex only under conditions of anaerobiosis(4). Under aerobic conditions no VEM formation is demonstrable and in addition an inactivation of its angiotropic properties occurs. The hypertensive kidney uniformly shows a specific alteration in VEM metabolism as a result of which VEM is formed under both aerobic and anaerobic conditions(5). This occurs *in vivo* as well as *in vitro*, the VEM being demonstrable in the renal vein and peripheral blood throughout the hypertensive syndrome. This metabolic defect is usually unaccompanied by any changes in overall oxygen consumption of the kidney. These alterations in renal VEM metabolism occur independently of any pathological changes demonstrable by the usual staining technics.

Materials and methods. The data here presented were obtained from normotensive and hypertensive rats, dogs and man. Hypertension was induced in the rat by perinephric caps on both kidneys, and in the dog by Goldblatt clamps on the renal arteries of both kidneys. Included in this series were kidneys from 6 control and 4 hypertensive rats, as well as 4 control and 3 hypertensive dogs. In the human subjects the renal cortical tissue consisted of biopsies obtained at operation from 2 hypertensives and 2 normotensives, and from 2 normotensive subjects very soon after death.

The tetrazolium studies were carried out as follows. Thin slices from the renal cortex were made as for microrespiration determinations and incubation performed within 15 minutes of receiving the specimen. To insure uniformity the sections were made at right angles to the long axis of the kidney. Two or 3 slices weighing on the average 150-200 mg were placed in an Erlenmeyer flask containing 5 ml physiological saline or Ringer phosphate (pH 7.4) and 0.5% TTC. The tissues were incubated at 37.5°C for 1 hour in air with constant shaking in the Warburg bath. Incubations were carried out with and without sodium succinate (0.4%), the latter substrate having been shown to intensify the TTC reduction and formazan deposition. The histochemical patterns were qualitatively similar with both solutions. At the end of 1 hour, the tissue slices were fixed in 10% neutral formalin. Frozen sections were made and mounted in glycerol for histochemical study. In accordance with the technic previously described, a portion of each set of slices was

1. Antopol, W., Glaubach, S., and Goldman, L., *Public Health Rep.*, 1948, v63, 1231.

2. Rutenburg, A. M., Gofstein, R., and Seligman, A. M., *Cancer Research*, 1950, v110, 113.

3. Black, M. M., Kleiner, I. S., and Speer, F., *Cancer Research*, 1950, v10, 204.

4. Shorr, E., Zweifach, B. W., Furchgott, R. F., and Baez, S., *Factors Regulating Blood Pressure*, Trans. 1st Conference, 1948, 32, Josiah Macy, Jr., Foundation.

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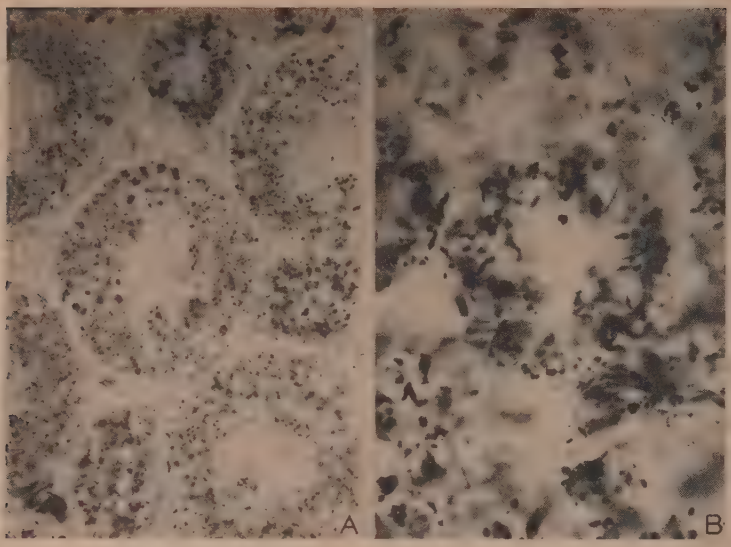


Normotensive Hypertensive

FIG. 1.

Kidney cortex of dog after incubation for 1 hour at 37.5°C in saline solution containing 0.5% tetrazolium (TTC) and 0.4% sodium succinate. Reduced TTC (formazan) deposited in cells. (a) Section from normotensive dog showing dust-like formazan granules in proximal convoluted tubules. (b) Section from hypertensive dog showing coarse plaques and clusters of formazan needles in proximal tubular cells. (300×).

extracted with acetone to remove the formazan for colorimetric determinations(6). The acetone extracted tissue was then dried in air and weighed to determine the color reading per mg of dried tissue. These values were related to oxygen consumption of slices from the same kidney as measured in Warburg manometers under similar substrate conditions.

Concurrent with the TTC studies, slices prepared from each kidney were examined for their ability to form VEM under aerobic and anaerobic conditions, utilizing the rat mesoappendix technic for the bioassay of VEM activity(7). The incubation procedures were as previously described(8).

Our previous studies have shown that prior anaerobic incubation of normal kidney tissue for 60 to 90 minutes will transform the *in vitro* VEM metabolism of the normal kidney to that of the hypertensive kidney. On subsequent restoration to oxidative conditions, the pretreated normal kidney now forms VEM in oxygen as well as in nitrogen. This procedure was also utilized in this study to determine whether this metabolic alteration might be associated with changes in the pattern of formazan deposition in these kidneys. Such experiments were carried out on the kidneys of 4 normal dogs, 1 normotensive human, and 6 normal rats.

Experimental results. (a) *Kidneys of Normal and Hypertensive Dogs.* The kidneys of normotensive dogs uniformly show a regular pattern of formazan deposition characterized by fine dust-like granules throughout the cytoplasm of cells of the proximal convoluted tubules (Fig. 1a). The nuclei remain unstained. The granules have a purplish cast and are almost entirely intracellular. Other tubular structures in the

6. Black, M. M., and Kleiner, I. S., *Science*, 1949, v110, 660.

7. Zweifach, B. W., *Methods in Medical Research*, V. R. Potter, Editor, 1948, v1, 131, Year Book Publishers, Chicago.

8. Shorr, E., Zweifach, B. W., Furchgott, R. F., and Baez, S., *Circulation*, in press.

TABLE I.

Total Reduced Tetrazolium (Formazan) and Oxygen Consumption Measured by Warburg Technic. Formazan (reduced TTC) expressed as color units per mg of acetone dried tissue per hour. These values may be expressed in terms of micrograms of reduced tetrazolium by comparison with a standard curve, *e.g.*, 133 = 4.7 γ, 121 = 4.2 γ, 96 = 3.2 γ, etc. Q_{O_2} = cc/g/hr wet weight.

Group	No.	Formazan Mean	Formazan Range	Q_{O_2} Mean	Q_{O_2} Range
		Dog			
Control	4	121	60-148	1.9	1.6-2.3
Hypertensive	3	118	107-129	2.3	1.8-2.7
		Human			
Control	4	96	79-123	1.6*	—
Hypertensive	2	106	—	1.5	—
		Rat			
Control	6	133	115-150	3.1	2.9-3.3
Hypertensive	4	121	87-157	2.8	2.6-3.2

* 2 subjects.

cortex also take up the formazan but less intensively and with a different pattern. The formazan deposits in the collecting and distal convoluted tubules are needle-like or polyhedral and have a more reddish hue. The glomeruli show minimal or no staining.

The staining characteristics of the hypertensive kidney are strikingly different, especially in the proximal convoluted tubules (Fig. 1b). Formazan deposition is much coarser with angular crystals, coarse needles and plate-like deposits in these cells. There is a distinct tendency for the dye to deposit extracellularly as well. The crystals are usually deposited towards the base and borders of the proximal convoluted tubular cells and may appear in the lumen of the tubules. There are no appreciable differences in staining of glomeruli, distal convoluted and collecting tubules.

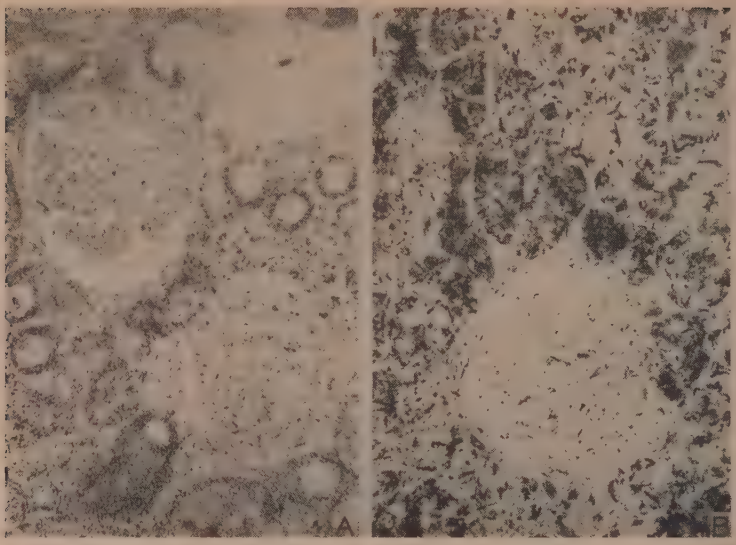
In spite of the differences in the staining pattern with TTC, the total amount of formazan per mg of tissue did not differ in normal and hypertensive kidneys, nor did the respiratory data reveal any significant differences between the oxygen consumption of the normal and hypertensive kidneys. (Table I) This was true for the kidney slices both in the presence and absence of succinate in the medium. An inference that can be drawn from the intensity of the stain in different parts of the kidney cortex is that maximal respiratory activity is localized in the proximal convoluted tubules.

Studies of the renal VEM mechanism gave results in conformity with our previous observations. VEM was formed by kidneys from normotensive dogs only under anaerobic conditions, whereas this vasoexcitor material was formed by the hypertensive kidney in approximately equal amounts under both aerobic and anaerobic conditions.

Thus, there is a parallelism between the disorganization in the proximal convoluted tubules of the hypertensive dog kidney indicated by the altered formazan pattern and the metabolic derangement as a result of which the hypertensive kidney loses its ability to restrict VEM formation to the anaerobic state. The possibility of a direct relationship between these two disturbances is under present investigation.

(b) *Kidneys from Normotensive and Hypertensive Human Subjects.* The same types of observations were carried out on biopsy specimens of renal cortex from kidneys of 4 normotensive and 2 hypertensive subjects. In one of the hypertensive subjects the occasion for surgery was a sympathectomy; in the other, an associated hydronephrosis. Of the 4 normotensive kidneys, 2 were obtained at operation, the specimens chilled immediately on removal and incubated within 30 minutes. The remaining 2 normotensive kidneys were obtained shortly after exitus. With these latter kidneys Warburg respiratory and VEM measurements were not carried out.

The results corresponded in every respect with those previously described for the dog. It is of interest that in both the hypertensive dog and human not all of the proximal convoluted tubules exhibited an alteration in the normal granular type of formazan deposition. In the dog, about 80% of the tubules were involved; in the human, about 60-70%. Sections of the normal and hypertensive human kidney under low power magnification (67×) are shown in Fig. 2 to illustrate the type of formazan distribution characteristic of both conditions. Under higher magnification the details are indistinguishable from



Normotensive Hypertensive

FIG. 2.

Human kidney cortex after incubation for 1 hour at 37.5°C in saline solution containing 0.5% tetrazolium (TTC) and 0.4% sodium succinate. (a) Normotensive patient; dust-like deposition of formazan; glomerulus unstained. (b) Patient with essential hypertension; coarse, heavy deposition of formazan in proximal convoluted tubules. Note tendency for dye to be deposited extracellularly as well. (67×).

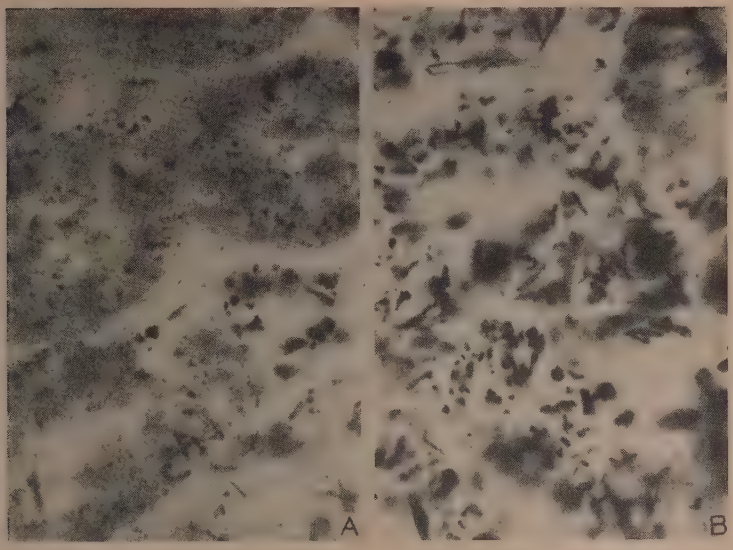
those seen in the dog in Fig. 1.

VEM production took place in both the hypertensive kidneys under aerobic and anaerobic conditions. The kidneys from normotensive patients formed VEM only under anaerobic conditions. The biopsy obtained from the hydronephrotic infected kidney exhibited considerable interstitial inflammatory reaction and had a low oxygen consumption. The other from the patient undergoing sympathectomy presented a normal histologic picture by the usual staining method and oxygen consumption was in the normal range for human kidney cortex. The total formazan content of the hypertensive kidney was also of the same order as that for kidneys from normotensive patients, despite a difference in the staining pattern visible under the microscope.

A biopsy from one of the normotensive kidneys came from an area adjacent to an infected cyst and showed an extensive interstitial inflammatory reaction. Nevertheless, the formazan pattern and VEM formation were of an entirely normal character.

(c) *Kidneys from normal and hypertensive rats.* The results on the kidneys of normal and hypertensive rats corresponded closely with those obtained in the dog and human. One precaution, prompt chilling and incubation, should be observed in carrying out these studies on the rat, in view of the greater susceptibility of rat kidney to anaerobiosis and of the consequent development of the metabolic and histologic alterations to be described.

(d) *Alterations in VEM metabolism and TTC staining of normal kidneys after anaerobic exposure.* We have previously demonstrated that after a prior exposure to anaerobiosis *in vitro* the normal kidney behaves like a hypertensive kidney in that it continues to form VEM when re-exposed to oxidative conditions(8). It was of interest to determine whether this metabolic derangement was accompanied by the development of formazan deposition similar to that observed in hypertensives. These experi-



Aerobic control After 90 min., anaerobiosis

FIG. 3.

Kidney cortex of dog before and after pretreatment under anaerobic conditions for 90 minutes. Treated with TTC and succinate as in previous illustration. (a) Usual appearance of formazan deposits in cells of proximal tubules; fine, dust-like and uniform. (b) After kidney has been pretreated under N_2 and returned to aerobic conditions. Formazan deposition transformed to pattern like that in hypertensive kidneys—coarse plaques, heavy needles. (300×).

ments were carried out on kidneys from 4 normal dogs, 1 normotensive human and 6 normal rats. In all instances the development of aerobic VEM formation was accompanied by the development of the coarse, plaque-like deposition of formazan crystals in the proximal convoluted tubules as illustrated in Fig. 3. This is indistinguishable from the pattern in the kidney of the hypertensive animal or human. The period of anaerobic exposure required for this transformation was 45 minutes for the rat, and 60-90 minutes for the human and dog. No differences in formazan deposition could be distinguished in the other structural units of the kidney.

The parallel appearance of an altered formazan pattern in the proximal convoluted tubules and of the derangement in VEM metabolism after prior anoxia suggests that the proximal convoluted tubules may be the site of VEM formation. A further extension of this inference would be that this defect in VEM metabolism might be related to the derangements in cellular intermediary metabolism of which the formazan deposition is a reflection. It should be mentioned that occasionally some of the proximal convoluted tubules in kidneys from normotensive subjects may show deviations from the normal type of formazan deposition. However, in no case of hypertension was the tubular pattern of formazan deposition normal.

Summary and conclusions. The incubation of slices of renal cortex with tetrazolium chloride (TTC) has disclosed patterns of formazan deposition in the proximal convoluted tubules with striking differences between normal and hypertensive kidneys obtained from dogs, rats and man. In the proximal convoluted tubules of kidneys from normotensive animals and man, the formazan is deposited as fine dust-like granules evenly distributed within the cytoplasm. In the hypertensive kidney the formazan is deposited in coarse clumps, plaques or needles, commonly at the cell margins and often extracellularly as well.

Despite the difference in formazan patterns the total amount of reduced TTC per mg of kidney tissue is approximately the same in normal and hypertensive kidneys, as is the oxygen consumption determined by the conventional micro-respiration technic. Concurrent studies of VEM metabolism show the uniform restriction of VEM production to anaerobiosis in normal kidneys, and the formation of VEM under both anaerobic and aerobic conditions by the hypertensive kidneys of animal and human subjects. When the VEM metabolism of a kidney from a normotensive rat, dog or human is transformed to the hypertensive type by a period of prior anaerobiosis *in vitro,* there is a parallel development of the characteristic hypertensive formazan pattern in the proximal convoluted tubules.

The reduction of TTC to the insoluble formazan results from an interaction between TTC and enzyme systems within the cell. The observations reported would indicate some alteration in the metabolic characteristics of the proximal convoluted tubules in experimental renal and essential hypertension. The association of these histochemical changes in the proximal convoluted tubules with alterations of the VEM mechanisms in experimental and human hypertension suggests, 1) that the proximal tubules may be the site of VEM formation, and 2) that the failure of the hypertensive kidney to limit VEM formation to anaerobiosis may be related to the enzymatic disorganization revealed by TTC.

Finally, the data presented provide specific evidence for a similarity between experimental renal hypertension and essential hypertension in man.

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Procarcinogenic Effect of Vitamin B_{12} on p-Dimethylaminoazobenzene-Fed Rats.* (18067)

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Diet is known to influence the carcinogenicity of p-dimethylaminoazobenzene (DAB). Riboflavin has been shown to exert a protective effect(1) while it has been reported that biotin is procarcinogenic(2). The experiments to be reported in this paper indicate that vitamin B_{12} exerts a marked procarcinogenic effect on DAB-fed rats.

Experimental. Weanling female Sprague-Dawley rats were fed a purified diet consisting of the following: isolated soybean protein,† 18 g; sucrose, 67.4 g; hydrogenated vegetable oil, 8 g; cod liver oil, 2 g; salt mixture(3), 4 g; choline chloride, 0.1 g; inositol, 10 mg; thiamine chloride, 1.5 mg; riboflavin, 0.5 mg; nicotinamide, 2 mg; calcium pantothenate, 1.0 mg; pyridoxine hydrochloride, 0.5 mg; vitamin K, 0.025 mg; biotin, 0.005 mg; folic acid, 0.5 mg; and p-dimethylaminoazobenzene, 70 mg. This diet contains approximately 0.27% methionine. One group of rats received this diet unsupplemented, one group received this diet plus 5 micrograms of vitamin B_{12} (Rubramin) per 100 g, a third group received this diet plus 0.6% DL-methionine, and a fourth group received the

* Research paper No. 910, Journal Series, University of Arkansas. This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

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2. du Vigneaud, V., Spangler, J. M., Burk, D., Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Science,* 1942, v95, 174.

† "Alpha Protein," obtained from the Glidden Company, Chicago.

3. Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition,* 1937, v14, 273.

TABLE I.
Influence of Vitamin B_{12} and Methionine on Incidence of Hepatomas in p-Dimethylaminoazobenzene-Fed Rats.

Supplement to diet	Avg daily food intake, g	Avg daily wt gain, g	Incidence of hepatomas, %	Liver wt*— % of body wt
None	6.4	0.1	17	9.3
Vitamin B_{12}	7.0	0.2	78	15.7
Methionine	8.1	1.2	11	7.0
Methionine + vit. B_{12}	10.5	1.2	33	11.0

* Liver weights include tumors, where present.

diet supplemented with both vitamin B_{12} and methionine. The rats were fed these diets for 170 days and were then killed and autopsied. The incidence of gross hepatomas was recorded. There were 9 rats in each group. Three rats in the unsupplemented group died without hepatomas during the experimental period. Two rats receiving vitamin B_{12}, one rat receiving methionine, and one rat receiving vitamin B_{12} plus methionine died with hepatomas during the experimental period. The incidence of hepatomas is based on those rats either surviving the 170-day period or dying with hepatomas.

Results and discussion. The pertinent data are presented in Table I. The addition of vitamin B_{12} to the DAB-containing, methionine-deficient diet increased the incidence of hepatomas from 17% to 78%. The addition of vitamin B_{12} to the methionine-containing diet increased the incidence of hepatomas only from 11% to 33%; thus methionine appeared to afford some protection.

The procarcinogenic effect of vitamin B_{12} may indicate that this vitamin is required for tumor growth or it may be a reflection of an influence of vitamin B_{12} on metabolic transformations of the carcinogen p-dimethylaminoazobenzene. In the former event these results might have general application to the entire field of cancer. Experiments are in progress to elucidate the mechanism of the procarcinogenic effect of vitamin B_{12}.

It would be unfortunate if the conclusion were drawn from these experiments that vitamin B_{12} itself is carcinogenic. There is no evidence that such is the case. On the contrary, a control group of rats receiving the soybean protein diet with vitamin B_{12}, but without p-dimethylaminoazobenzene, showed no hepatic tumors.

Summary. Vitamin B_{12} has been found to enhance markedly the carcinogenic effect of p-dimethylaminoazobenzene in rats receiving a methionine-deficient diet. However, a control group of rats receiving this diet without p-dimethylaminoazobenzene showed no hepatic tumors.

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Life Maintaining Activity of Δ^1 Desoxycorticosterone Acetate.* (18068)

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Life maintaining activity in adrenalectomized rats has been obtained with Δ^1 allopregnen-21-ol-3, 20-dione 21 acetate for which the trivial name Δ^1 desoxycorticosterone acetate was suggested(1). This steroid has been compared with desoxycorticosterone acetate (Percorten, Ciba) for life maintaining

* This investigation was done under contract with the Office of Naval Research, Navy Department.

1. Djerassi, C., Scholz, C. R., and Leathem, J. H., *Experientia*, 1949, v5, 204.

TABLE I.
Survival of Adrenalectomized Immature Male Rats Treated with Desoxycorticosterone Acetate or Δ^1 Desoxycorticosterone Acetate.

Daily treatment mg	No. of rats, ADX	No. rats surviving at 10 days	15 days	20 days	Avg body wt increase in 20 days, g
		Desoxycorticosterone acetate			
0.025	10	7	4	4	+20
0.050	10	10	10	9	+48
0.100	10	10	10	10	+69
		Δ^1 Desoxycorticosterone acetate			
0.10	9	7	2	0	—
0.25	11	11	11	7	+23
0.50	10	10	8	8	+45
None	8	0			—

and body weight increasing activity.†

Male rats of the Long-Evans strain were used when approximately 60 g in body weight. The rats were bilaterally adrenalectomized and fed *ad libitum* a diet consisting of 18% casein (Labco), 60% dextrose, 10% yeast, 5% Wesson's salt mixture, 5% Mazola and 2% cod liver oil. The steroids were dissolved in peanut oil and injected subcutaneously once daily.

Ten normal male rats gained an average of 69 g body weight in 20 days whereas untreated adrenalectomized rats lost weight and died 4 to 8 days after the operation. Daily injections of peanut oil alone (0.2 cc) did not alter survival time. The pertinent data obtained with the steroids are presented in Table I and indicate that Δ^1 DCA is about 1/8 to 1/10 as potent as DCA. 0.25 mg and 0.50 mg of Δ^1 DCA daily permitted good survival and approximately 1 and 2 g gain in body weight daily respectively. Normal body weight increases and 100% survival was obtained with 0.1 mg DCA daily. Thayer (2) has previously shown that 0.1 mg of DCA is necessary to permit normal growth and complete survival in immature adrenalectomized rats. For 80% survival and 1 g increase in body weight daily, dosages of 20 μg(2) and 67 μg(3) of DCA have been reported. Our data with DCA indicate that an 80% survival is associated with a 2 g daily body weight gain.

Cessation of Δ^1 DCA administration after 20 days of 0.5 mg daily resulted in death 6 and 9 days later with 2 rats. Discontinuing DCA after 20 injections of 0.025 mg resulted in death in 3 rats after 3 days whereas after 0.05 mg daily, 6 rats died 5, 6, 8, 9, and 10 days after the last injection.

The livers from normal and adrenalectomized rats maintained for 20 days on both steroids were dried to constant weight at 95°C to determine water content. Groups of 4 to 7 rats were studied and liver water content was normal in all cases. The dried livers from two groups of adrenalectomized rats on Δ^1 DCA for 20 days and from normal rats were analyzed for total nitrogen and the data converted to protein by the factor 6.25. Adrenalectomized rats maintained on 0.25 mg and 0.50 mg of Δ^1 DCA had liver protein values of 0.85 g and 0.87 g/100 g body weight respectively. The livers from normal rats contained an average 0.84 g protein/100 g body weight and indicated that relative liver protein content was the same in both groups.

To observe any organ weight changes which Δ^1 DCA might induce, 0.5 mg was injected subcutaneously for 10 days into five 22-day-old female rats and the organ weights compared with an equal number of littermate controls. Weights of the hypophysis, adrenal, thyroid, ovary, uterus, thymus, heart, spleen and kidney were unchanged. Liver weight

† The steroids were generously supplied by Ciba Pharmaceutical Products, Summit, N. J.

2. Thayer, S. A., *Vitamins and Hormones*, 1946, v4, 311.

3. Kuizenga, M. H., Nelson, J. W., and Cartland, G. F., *Am. J. Physiol.*, 1940, v130, 298.

was somewhat greater in the injected rats being 5.3 g/100 g body weight as compared with 4.6 g/100 g body weight in controls.

Summary. The steroid Δ^1 desoxycorticosterone acetate will maintain adrenalectomized immature rats for 20 days with dosages of 0.25 mg and 0.5 mg and will increase daily body weight 1 and 2 g respectively. Activity is approximately 1/8 to 1/10 that of desoxycorticosterone acetate. Liver water content was unchanged in maintained adrenalectomized rats and the relative liver protein content of adrenalectomized rats receiving Δ^1 desoxycorticosterone acetate was comparable to that of normal rats. This steroid did not alter organ weights in normal immature female rats over a 10-day period except for a tendency toward liver weight increase.

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Growth Requirements of *Streptococcus mitis* and Sulfonamide Resistance.* (18069)

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Previous work in our laboratory showed that trained sulfathiazole-resistant strains of *Streptococcus mitis* differed considerably in other biochemical properties from the original strains(1,2). The resistant strains acquired the ability to grow on 40% bile agar, in 6.5% NaCl, in .1% M.B. milk, and in the presence of increased concentrations of 4 antibiotics. These properties are characteristic of enterococci(3), and were shown by a known strain of *Streptococcus fecalis,* No. 9790, obtained from the American Type Culture Collection.

The growth requirements of an organism are fundamental properties sometimes suggesting a possible mechanism for their response to bacteriostatic agents. Since we had observed that the resistant strains grew readily in a medium containing no riboflavin (Bacto Riboflavin Assay Medium), while the parent strains did not, we have investigated the vitamin requirements of several parent and resistant strains of *S. mitis* and of *S. fecalis,* No. 9790. This paper reports the results of that study.

Experimental. Semi-synthetic medium. This was a modification of the medium used by Smiley, Niven, and Sherman in their study of the growth requirements of *S. salivarius*(4). It was prepared in two stages, Part A being autoclaved prior to addition of Part B. Each batch of medium, upon the addition of the vitamins, was filtered through a Seitz filter.

Organisms used. Five strains of streptococci showing the biochemical characteristics of *Streptococcus mitis,* isolated in our labora-

PART A

Casein hydrolysate, 10% solution*	50 ml
DL-Tryptophane	60 mg
Dipotassium phosphate	6.0 g
Glucose	5.0 g
Sodium thioglycollate	100 mg
" chloride	2.0 g
Magnesium sulfate, cryst.	80 mg
Ferrous sulfate, cryst.	4 mg
Manganese chloride	1.2 mg
Uracil	10 mg
Water to	1,000 ml
pH 7.5	

* Vitamin-free casein hydrolysate (acid) 10% solution prepared by Nutritional Biochemicals Corp., Cleveland, Ohio.

* A portion of the data presented in this paper was taken from a thesis submitted in June, 1950, by Virginia M. Harrison to the University of Colorado in partial fulfillment of the requirements for the degree Master of Science.

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PART B	Amt per 250 ml of above
Thiamine hydrochloride	100 gamma
Pyridoxine hydrochloride	100 "
Calcium pantothenate	100 "
Riboflavin	100 "
Nicotinic acid	100 "
Biotin	1 "
Pteroylglutamic acid	1 "
p-aminobenzoic acid	10 "
Xanthopterin	1 "

tory, and these strains trained to sulfathiazole resistance, were used. Training was accomplished by repeated transfer in tryptose phosphate broth containing gradually increasing concentrations of sodium sulfathiazole. In addition another *mitis* strain maintained as a stock culture and *S. fecalis* No. 9790 were included. The letter S designates the trained sulfathiazole resistant strains.

Procedure. Tubes containing 5 ml of the medium with all the vitamins or with one of 8 vitamins omitted, were inoculated with 1 drop of a twice-washed suspension from a 24-hour culture in tryptose phosphate broth, made up in saline to a turbidity matching a No. 5 McFarland standard. All tubes were incubated at 37° for 48 hours, after which time the cultures were transferred to Klett-Summerson colorimeter tubes and the turbidity determined. Positive controls contained the complete medium inoculated with the organism under investigation. Blanks containing the uninoculated medium were measured for turbidity and this value subtracted from those tubes showing growth. The percentage of growth achieved in a vitamin-deficient medium was calculated by the following formula:

$$\% \text{ of growth} = \frac{\text{Growth in vitamin-deficient medium—Blank}}{\text{Growth in complete medium—Blank}}$$

Averages of the readings of 2 identical tubes were used to determine each value. The determinations were repeated 3 times for each organism and each vitamin.

Inhibitory concentrations of sulfathiazole were determined by inoculating tubes of the complete medium containing 10, 1, .1, .01, .001, .0001, .00001, .000001, and 0 mg per ml of sulfathiazole. One drop of a 24-hour broth culture was used to inoculate and the reading was made at the appearance of growth in the control tubes. The concentration of sulfathiazole which prevented discernible turbidity was called the inhibiting concentration.

Results and discussion. The inhibiting concentrations of sulfathiazole in the semi-synthetic medium of the 12 strains studied are shown in Table I. All the trained strains are at least 1,000 times as resistant as the parent strains. The concentration necessary for inhibition of these resistant strains is the same as that required for *S. fecalis* No. 9790.

Table II is a summary of the percentage of growth achieved by 5 organisms in the absence of one of 8 vitamins as compared to their growth in the complete medium. The values represent the average of 3 separate determinations, each of which was performed in duplicate. A value which shows less growth in the deficient medium showed this decrease in all 3 determinations. Individual values never varied more than 15% and usually much less.

The 3 *mitis* strains showed no appreciable growth in the absence of calcium pantothenate and nicotinic acid. All were inhibited by the absence of PABA, pyridoxine, and biotin, while 2 of the 3 strains required riboflavin and one thiamine for complete growth. All 3 strains achieved maximum growth in the absence of pteroyl glutamic acid (PGA). The growth response of the trained resistant strain 6 S to all of the vitamins is the same as that for *S. fecalis* No. 9790. Omitting thiamine, riboflavin, and PABA had no effect on the growth of these sulfathiazole resistant strains. Incomplete growth was observed in

TABLE I.
Inhibitory Concentration of Sulfathiazole Expressed as mg/ml.

Organism	Sensitive parent	Resistant mutant
1*	0.0001	1.0
4	0.0001	1.0
6	0.0001	1.0
17	0.0001	1.0
34	0.001	1.0
B	0.0001	—
9790	—	1.0

* 9790 = *S. fecalis;* all others = *S. mitis.*

TABLE II.
Comparison of Percentages of Full Growth Achieved by Each Organism in the Vitamin-deficient Media.

Organism	No thiamine, %	No riboflavin, %	No PABA %	No calcium pantothenate, %	No nicotinic acid, %	No pyridoxine HCl, %	No biotin, %	No PGA, %
34	100.0	23.7	67.5	0	0	27.4	37.7	97.6
B	58.9	26.1	47.6	3.3	0	32.8	47.6	95.2
6	87.1	85.4	67.9	2.6	2.8	33.7	35.4	97.2
6S	97.1	95.5	97.3	1.4	28.3	61.8	34.7	0
9790	97.1	96.5	98.7	1.8	25.8	61.8	44.8	0

Organisms 6, 34 and B = *S. mitis.*
6S = Trained resistant strain from 6.
9790 = *S. fecalis.*

the absence of nicotinic acid, pyridoxine, and biotin, and no growth in the absence of calcium pantothenate, quite like the response shown by the *mitis* strains to these vitamins. The growth response to PGA, however, was quite different than that shown by the *mitis* strains. The strains of *S. mitis* do not need PGA, while the trained resistant strain and the strain of *S. fecalis* do. Niven and Sherman (5) have reported nicotinic acid, pyridoxine, biotin, and pantothenate as necessary to growth for 5 of 5 strains of *S. fecalis* studied, and folic acid necessary for 3 of the 5 strains.

To test the possibility that this loss of ability to synthesize PGA was a property common to other trained sulfathiazole resistant strains of *S. mitis*, 4 more strains and their resistant mutants were examined for their PGA requirements. The results, summarized in Table III, show that these additional strains responded in the same manner as did the first strains tested. It was interesting that a variant of strain 34, trained to only 10 times the resistance of the parent strain, showed the same responses to PGA as the parent strain; but when the resistance was carried on to 1,000 times, this new strain required PGA, as shown in the table.

TABLE III.
Comparison of the Percentages of Full Growth Achieved by Each Organism in PGA-deficient Media.

Organism	No PGA, %
1*	98.1
1S	5.3
4	100.0
4S	5.6
17	100.0
17S	5.9
34	100.0
34S	16.3

* Organisms 1, 4, 17, and 34 = *S. mitis.*
Organisms 1S, 4S, 17S, and 34S = trained resistant strains.

Lampen and Jones(6) demonstrated that *L. casei* and *S. fecalis R.* require PGA for growth, and that they were virtually unaffected by high concentrations of sulfadiazine. They concluded that sulfonamides block the synthesis of PGA from PABA. Nimmo-Smith, Lascelles, and Woods(7) further substantiated this hypothesis by showing that resting cell suspensions of *Streptobacterium plantarum* synthesized folic acid, and this synthesis was inhibited by sulfonamides. Our data would indicate that susceptible *S. mitis* strains, trained to sulfonamide resistance, become resistant because the resistant strains no longer synthesize PGA, but can utilize it preformed from the medium. The synthesis but not the utilization is blocked by sulfonamides. While this is not a new mechanism, this is the first time, to our knowledge, that it has been shown to be associated with trained sulfa-resistant strains of streptococcus. It is also rather interesting that all of the properties of the resistant strains that we have investigated, including the growth response to vitamins, have been entirely the same as those for *S. fecalis.*

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6. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, 1946, v166, 435.

7. Nimmo-Smith, R. H., Lascelles, J., and Woods, D. D., *Brit. J. Exp. Path.*, 1948, v29, 264.

Summary. 1. Differences in the vitamin requirements of susceptible strains of *S. mitis*, their artificially-induced resistant variants and a naturally-resistant *S. fecalis*, have been determined. 2. The greatest difference was in the PGA requirements, the *mitis* strains grew in its absence; the resistant mutants required PGA. 3. The induced resistance of the trained *S. mitis* strains appears to be the same as that of the natural resistance of *S. fecalis*, and may be due to the loss of ability to synthesize PGA, the synthesis of which is inhibited by sulfonamides.

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Effect of Method of Homogenization of Beef Muscle Tissue on Activity of Succinic Dehydrogenase System.*† (18070)

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In the present studies, application of the method for determining the succinic dehydrogenase activity of rat tissues(1,2) to studies of the activity of this system in beef muscle was investigated. In previous work the rat muscle tissue usually has been prepared for assay in a Potter-Elvehjem homogenizer. Since the 1-2 g samples used in this procedure would not be sufficient for a representative sample of beef muscle tissue, alternative methods of tissue preparation were investigated. The results obtained with the use of the Waring blendor for varying periods of time, for tissue minced either with scissors or with a Latapie mincer prior to homogenization in the Waring blendor, the use of the Potter-Elvehjem homogenizer, and grinding with sand as techniques for preparing beef and rat tissues to determine succinic dehydrogenase activity are reported in this paper.

Experimental. The method as described by Schneider and Potter(1,2) was used for the manometric determination of succinic dehydrogenase activity. The results are expressed as μl O_2 uptake per hour per 100 mg wet weight of beef muscle or per mg dry weight of rat tissue.

For beef muscle, 10% homogenates of 5 g or 10 g samples were prepared in the Waring blendor (500 ml capacity), and 3 levels of tissue were assayed for each homogenate. Low temperatures were maintained during the homogenization with ice water, and the use of a Varitran afforded constant speed of homogenization. For the first experiments, a 5-minute homogenization period was used with a 5 g sample which was minced with a scissors before homogenization. Although the values for 3 levels of tissue from one homogenate checked well, difficulties of reproducing results with separate homogenates were encountered. The use of larger samples gave no improvement in reproducibility.

Shorter homogenization periods were investigated and were found to result in better reproducibility and higher values. For 3 separate samples of *Longissimus dorsi* and *Semitendinosus* muscle, a 2-minute period was compared to a 5-minute period and the values averaged 145 and 57 μl of O_2 uptake per 100 mg of fresh tissue per hour, respectively. Further studies were undertaken to compare three homogenization times, namely

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† Journal Paper No. 27, American Meat Institute Foundation.

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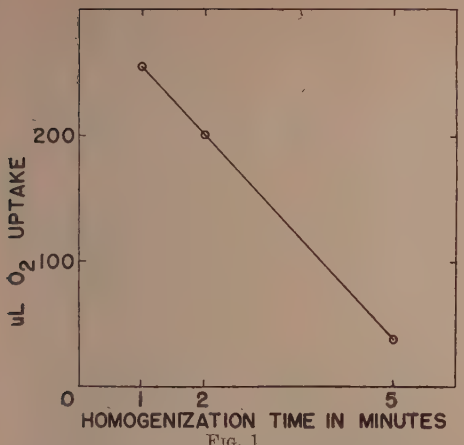

FIG. 1.

Effect of homogenizing beef muscle in the Waring blendor for varying periods of time on the succinic dehydrogenase activity (expressed as O_2 uptake per 100 mg wet weight per hour).

1, 2, and 5-minute periods. Representative results, obtained for several samples of beef round, are shown in Fig. 1. It will be seen that some decrease in activity occurred when the beef muscle was homogenized for 2-minutes as compared to 1 minute and a striking decrease was observed after homogenizing for 5 minutes in the Waring blendor. The 5-minute period was then compared to a 1-minute period using 10 g samples, since it was thought that the increase in volume of homogenate which resulted when the sample size was doubled would decrease the possibility of inactivation of the enzyme system by aeration during homogenization. For 8 *Longissimus dorsi* muscles of beef, the oxygen uptake for 1-minute homogenates averaged 153, and the oxygen uptake for 5-minute homogenates averaged 36. Similar data were obtained for 3 *Semitendinosus* muscles with an average of 164 for the 1-minute period and 42 for the 5-minute period, or a ratio of approximately 4:1.

Scissor minces were not homogenized in the Waring blendor for periods shorter than 1 minute because of inadequate tissue fragmentation and difficulties of sampling the preparation. A finer mince was obtained with the Latapie mincer. This mince was not fine enough for a uniform suspension without further treatment; therefore homogenizing for ½, 1, and 1½-minutes in the Waring blendor was investigated. Considerable loss in activity was noted for homogenization periods that exceeded ½ minute for the 3 samples of beef muscle studied. For example, values of 157, 84, and 46 were observed with the Latapie mince which had been homogenized in the Waring blendor for ½, 1, and 1½ minutes, respectively; while for a 1-minute Waring blendor treatment of a scissor mince of the same sample, the value was 156. Similar results were obtained in other experiments. It was concluded that a 1-minute homogenization (Waring) of the scissor mince was as effective in retaining succinic dehydrogenase activity as homogenizing the Latapie mince in the Waring blendor for ½ minute. Since the homogenization of beef muscle scissor mince for 1 minute in a Waring blendor was the most satisfactory method studied, it was desirable to compare the results for rat tissues obtained with this method with those obtained with the Potter-Elvehjem homogenizer and grinding in sand.

Homogenates of rat tissues were prepared with the Waring blendor for 1, 2, or 5-minute periods and the data obtained compared with those obtained with the Potter-Elvehjem homogenizer and sand grinding technics. Two gram samples of rat muscle were taken, minced with a scissors and homogenized as described. Two per cent homogenates were prepared in all cases. The results are presented in Fig. 2 (2-7 analyses were made for

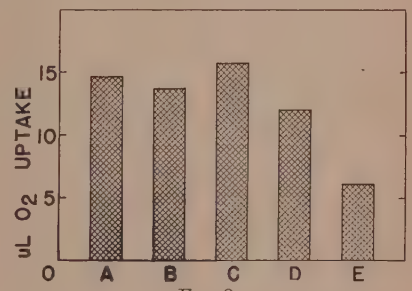

FIG. 2.

Succinic hydrogenase activity of rat muscle per mg dry weight per hour determined after different methods of homogenization of the tissue. A. Ground in sand. B. Potter-Elvehjem homogenizer. C. Homogenized 1 minute in the Waring blendor. D. Homogenized 2 minutes in the Waring blendor. E. Homogenized 5 minutes in the Waring blendor.

each sample treatment). Slightly higher values were obtained with the Waring blendor when a homogenization time of 1 minute was used, as compared to the values obtained by the Potter-Elvehjem homogenizer or grinding with sand. The 1-minute Waring homogenates had higher succinic dehydrogenase activity than 2 and 5-minute homogenates which is in agreement with the data for beef muscle.

Parallel experiments were conducted with rat liver by the use of 1 g samples for homogenization and it was found that the sand-ground and Potter-Elvehjem homogenized samples had somewhat greater succinic dehydrogenase activity than 1-minute Waring homogenates, 63 μl O_2 uptake per mg dry weight per hour, as compared to 45. This may be explained by the fact that liver is easily fragmented and aeration and inactivation probably occur more rapidly during homogenization than during similar treatment of muscle tissue. Longer homogenization (Waring) periods resulted in a decrease in succinic dehydrogenase activity.

Discussion. Quinlan-Watson and Dewey (3) reported considerable inactivation of cytochrome oxidase of guinea pig liver after 1-minute homogenization in the Waring blendor at room temperature. As noted in the present studies, the loss in succinic dehydrogenase activity of rat liver occurred more rapidly than in muscle tissue. Cytochrome c oxidase is present in large excess of succinic dehydrogenase in tissues and some inactivation of cytochome oxidase may not affect the activity of the succinic dehydrogenase system as assayed in the present experiments(1).

The inactivation of wheat germ enzymes by 2 and 5-minute Waring blendor treatment was cited by Stern and Bird(4) since sand-ground suspensions retained more enzymatic activity. In our studies the sand-ground suspensions of rat muscle did not have higher succinic dehydrogenase activity than 1-minute Waring blendor homogenates.

For demonstrating the presence of the enzyme system in tissues, or the necessary components of the system, the use of technics which result in some inactivation of the enzyme, such as homogenization for 2 or 5 minutes with the Waring blendor would probably not negate the validity of the findings. However, for studying the activity and stability of the system in muscle tissue on a quantitative basis, investigations on the stability of the enzyme system during homogenization would appear to be a necessary prerequisite.

Summary. The succinic dehydrogenase activity of beef muscle determined after different methods of homogenization of the tissue was investigated. The amount of beef muscle needed for a representative sample was too large to be conveniently used with the Potter-Elvehjem homogenizer; however, the samples could be prepared for assay by homogenizing in a Waring blendor, provided that the homogenization time is held to a minimum. This was evidenced from studies which showed that homogenizing the minced beef tissues in a Waring blendor for 1 minute or homogenizing Latapie minces for ½ minute resulted in a greater succinic dehydrogenase activity than that obtained when the tissues were homogenized for longer periods. Other experiments with rat tissues showed that the enzyme activity of rat muscle homogenized in the Waring blendor for 1 minute, in a Potter-Elvehjem homogenizer, or ground with sand was essentially the same.

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Normal Hemoglobin Clearances in Chronic Proteinuria.* (18071)

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(Introduced by William Dock.)
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The mechanism of proteinuria has evaded a satisfactory explanation. Proteinuria may be due to renal or extra-renal factors, such as plasma with abnormal protein, *e.g.*, hemoglobin or Bence Jones protein which pass through normal kidneys into the urine. If protein is due to renal factors (organic damage with or without change in renal hemodynamics), one is confronted with a number of possible explanations: (1) primary or secondary glomerular change which allows the free passage of protein through the glomerular tuft; (2) faulty reabsorption of protein from an ultrafiltrate with a normal protein content; (3) combinations of (1) and (2). There is no evidence that tubular excretion plays any role in the production of proteinuria. That the basic difficulty may be extra-renal should be considered since alterations in protein metabolism or vascular dynamics may be associated with proteinuria without demonstrable changes in the kidney. These extra-renal factors may also contribute, in the damaged kidney, to produce or aggravate proteinuria.

In order to evaluate the role of the glomerular membrane in the production of proteinuria, it was decided to use the hemoglobin molecule (approximately the same molecular weight, size and shape as albumen) as a test substance and to measure its renal clearance simultaneously with inulin, in normal subjects and those with proteinuria. A ratio so obtained has been termed the glomerular permeability by Monke and Yuile(1).

Methods and procedure. Five convalescent patients with no history or evidence of renal damage and without proteinuria were used as a control group. A group of 3 patients with the nephrotic syndrome (5 to 20 g/day of proteinuria) were used as the test group. All patients were tested at least 10 hours after their last meal and hydrated with 500-1000 cc of water by mouth prior to the test. The usual procedure for renal clearances was followed. The urine collection periods varied from 8-30 minutes, depending upon urine flow through an indwelling urethral catheter. Bladder evacuation was assured by the washout, and air injection and expression. Continuous infusion was used throughout with a priming infusion of 75-100 cc of 6% hemoglobin solution‡ and 30 cc of 10% inulin; sustaining infusions contained 3% hemoglobin and a proper amount of inulin using 5% glucose in water as the diluent. The rate of the sustaining infusion was regulated at 4 cc per minute by the use of a Harvard tunnel clamp. Concentrations of inulin in plasma and urine were determined by Dische's method as modified(3), and determination of hemoglobin concentrations in plasma and urine by the method of Turner(4) at a wavelength of 545 λ on a Coleman spectrophotometer.

Results and discussion. Table I lists the data obtained from 5 normals and 3 patients with the nephrotic syndrome. The data represent successive clearance periods in each patient following the appearance of hemoglobinuria.

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† Postdoctorate Fellow, U.S.P.H.S.

‡ Supplied through the courtesy of Dr. Gilbert Bayne of Sharp & Dohme, and is the same as the solution described by Pennell.(2)

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TABLE I.
Renal Handling of Hemoglobin.

Patient*	Age	Diagnosis	C_I	C_H	C_H/C_I
C.W.	67	A.S.H.D.	80.5	2.57	3.19
			61.8	2.10	3.40
			69.2	2.69	3.88
			62.5	2.39	3.85
			61.6	2.83	4.59
F.J.	26	Conv. pneumonia	114.5	1.94	1.69
			105.9	2.38	2.25
			114.7	2.82	2.46
			103.6	3.03	2.94
			115.8	3.53	3.04
			78.1	2.43	3.1
T.J.	34	Bronchiectasis	102.0	2.32	2.27
			111.2	3.02	2.72
			108.3	3.19	2.95
			101.8	2.54	2.52
			73.1	1.86	2.55
M.W.	46	Conv. pneumonia	93.4	1.51	1.61
			91.5	2.37	2.59
			111.5	3.07	2.75
			82.0	2.98	3.63
			99.8	3.39	3.40
M.W.	25	Psychoneurosis	107.8	1.16	1.07
			88.8	1.18	1.33
			119.5	1.75	1.46
			102.6	1.65	1.61
A.W.	24	Nephrotic syn.	21.8	0.67	3.07
			23.4	0.58	2.48
			19.4	0.75	3.87
H.M.	38	Nephrotic syn.	46.3	0.43	0.93
			42.3	0.61	1.44
			33.1	0.60	1.81
			30.1	0.67	2.23
H.L.	65	Nephrotic syn.	12.7	0.22	1.73
			21.5	0.35	1.63
			18.0	0.34	1.89
			17.2	0.39	2.27
			20.2	0.61	3.02

* All males.

In carrying out the above clearance procedures it soon became apparent that the time of appearance of hemoglobinuria was extremely variable in both the normals and proteinuric group. It was decided to plot all ratios (CH/CI) against time, establishing the time of appearance of the first pink or red urine (hemoglobinuria) in each patient as zero time (Fig. 1).

It has long been assumed that the presence of large amounts of protein in the urine of patients with the nephrotic syndrome is associated with alterations in the capillary permeability and an increase in the size of these capillary pores which will allow the passage of molecules the size of albumen. If one assumes that there is a point reached following the appearance of hemoglobinuria when the tubules are either saturated or reabsorbing hemoglobin at a maximal rate; then what appears in the urine is proportional to the amount of hemoglobin appearing in the glomerular filtrate. In the normal, the maximum ratio CH/CI appears to be within the range of 2%-5%, at blood levels up to 550 mg % maintained for over one hour (Fig. 1).

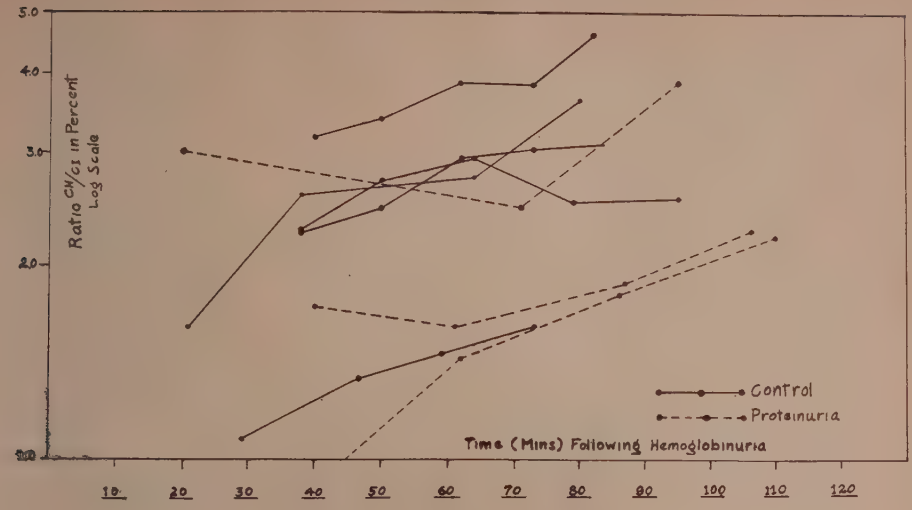

FIG. 1.

It does not rise with further increases in the plasma level of hemoglobin, but may increase with long sustained infusions. Assuming that proteinurics have larger and more numerous "pores" the size of albumen molecules the curves of the nephrotic (proteinuric) patients would necessarily fall in a range considerably above the range as plotted for normals, since hemoglobin is no larger than albumen and escapes normally at plasma levels one-fortieth the albumen concentration. As the curves in Fig. 1 indicate, one is forced to the conclusion that with any particular range of filtering bed in the controls and proteinurics their respective kidneys handle hemoglobin molecules in a similar fashion, if anything the hemoglobin clearances tend to be low in chronic proteinuria.

The data as presented seem to agree very well with the inferences drawn from the observations of Corcoran(5) and his co-workers who carried out similar observations with levan (M.W. 8000). Similarly, Rytand and Rantz(6) have shown that the anti-streptolysin clearances (globulin) of nephrotics follow a logarithmic increase as compared with an arithmetic increase in the quantity of protein excreted in the urine. These observations would tend to lend support to the premise, as suggested by the work described herein, that simple physical porosity of the glomeruli is not increased over normal in nephrotic patients excreting large amounts of albumen in their urine.§ Permeability to large molecules, with large electrical charges, appears to increase far more than that to smaller molecules such as levan.

Conclusions. 1. The ratio of simultaneously determined renal clearances of hemoglobin and inulin, in successive clearance periods in a group of 5 control and 3 patients with the nephrotic syndrome, indicate that the overall glomerular porosity, for hemoglobin, of the patients with proteinuria is no greater than that of normals.

2. It is probable that glomerular permeability, in terms of "pores," is not the factor determining the degree of proteinuria in the nephrotic syndrome.

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§ Observations similar to those reported here for the nephrotic patients have been made in one case of multiple myeloma and two patients with vascular disease, all associated with marked proteinuria.

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Alkaline Tide of the Alligator. (18072)

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In a study of the biochemistry of *A. mississipiensis* in the fasting state(1) a reference was made to the magnitude of the variation in certain blood constituents which occurred after feeding the animals. It was deemed advisable to study this phenomenon and report the results in some detail.

The animals thrive in captivity and through all of the warmer months they desire large amounts of food every 3 or 4 days. It is not uncommon for them to consume as much as one-fourth their body weight at one meal. Blood glucose and electrolyte studies indicate that about 3 days are required for the complete digestion of any one meal after which time the blood constituents are again at fasting levels. Although the individual variations are considerable, available evidence indicates that the food remains in the stomach from 24 to 36 hours. The plasma becomes lipemic during the second day and the lipemia is usually absent by the sixtieth hour after feeding. During the period of gastric digestion the bolus is subjected both to the action of the muscles of the stomach wall and to a large volume of hydrochloric acid at a pH of 2 or less. The secretion of this acid produces the typical qualitative picture of an "alkaline tide" but the duration and intensity of this phenomenon is far greater than that reported for the human(2,3). Although the general picture of the electrolytes of the fasting alligator is quite similar to that found in the human, the plasma changes after feeding are so great that the pH often becomes as alkaline as 7.8 and a few values of over 8 have been observed. Along with the marked rise in pH the chloride falls to a low level and the bicarbonate rises to a correspondingly high level. In one instance the chloride level of an animal declined from the normal fasting level to 7 m.eq./liter. Plasma bicarbonates as high as 88 m.eq./liter have also been observed.

Eleven alligators of undetermined sex from 3 to 5 years of age and weighing from 1.5 to 7.0 kg were used in the present study. Six smaller alligators weighing 85.8 to 177.5 g were ashed to determine their composition. The analytical methods employed for the estimation of plasma pH, CO_2, chloride, sodium, calcium, and phosphorus were reported in an earlier report on the results of blood and urine analyses(1). A limiting factor in all of the experiments was the large amount of blood required for the different simultaneous analyses, and several experiments had to be concluded prematurely to avoid severe anemia in the animals.

The following procedure was used in the acid-base studies. Blood was obtained by cardiac puncture using a No. 19 gauge needle and a 10 ml hypodermic syringe. About 2 ml of blood were drawn into the syringe, the syringe was detached with the needle left in the heart, and a Beckman blood glass electrode was quickly inserted in the needle. About one-half ml of blood was allowed to well up in the electrode chamber, the needle was withdrawn from the heart, and the pH was determined within 20 seconds. The temperature of the alligator is within 2°C of room temperature which enables one to determine the pH directly without making any correction for temperature changes. Although it was not found necessary to cover the heparinized blood specimens for the sodium, chloride, calcium, or phosphorus determinations, the specimens for CO_2 analyses were placed under oil immediately and the total plasma CO_2 content estimations were done as soon as possible.

Table I presents a more or less typical

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TABLE I.
Changes in Plasma Composition Following Feeding.

	Fasting				9 hr				19 hr			
No.	pH	Cl meq/l	CO_2 mM/l	Na meq/l	pH	Cl meq/l	CO_2 mM/l	Na meq/l	pH	Cl meq/l	CO_2 mM/l	Na meq/l
3	7.36	111	19.4	151	7.67	93	34.6	144	7.64	96	37.7	146
4	7.36	115	15.3	153	7.56	96	26.2	148	7.47	92	35.4	150
6	7.34	112	20.1	151	7.83	88	44.6	149	7.75	47	88.6	150
7	7.32	109	19.9	151	7.48	100	27.4	144	7.26	99	29.4	147
8	7.32	113	21.1	151	7.96	76	45.5	148	7.56	87	37.1	144
9	7.33	102	25.2	150	7.33	93	29.8	153	7.52	88	32.2	152
11	7.30	107	24.1	151	7.56	91	30.1	144	7.41	90	32.0	151
13	7.58	87	30.2	151	8.09	49	74.1	147	7.69	72	44.0	150

	24 hr				180 hr			
3	7.64	98	38.8	150	7.38	115	19.4	152
4	7.60	93	36.3	155	7.21	117	14.3	148
6	7.53	81	40.8	152	7.22	113	17.6	151
7	7.43	99	26.7	150	7.27	109	19.2	153
8	7.57	89	25.7	154	7.29	115	19.7	154
9	7.59	81	41.2	148	7.17	109	26.9	150
11	7.57	92	34.0	150	7.45	99	26.3	144
13	7.53	70	48.0	150	7.57	87	39.3	147

picture of the "alkaline tide" that follows the ingestion of food (whole rats) by the alligator. It will be noted that the individual differences are considerable. Alligator No. 7 showed the least response and No. 6 showed the greatest response. At the time the high blood pH (8.09) was observed in No. 13, the alligator was placed under careful observation to ascertain the effect of extreme alkalemia on respiration, irritability, etc. The animal appeared to be normal in all respects. Curiously enough some alligators show a perennial alkalemia, and there is no evidence that these animals are in poor health or that the continued alkalinity is in any way injurious. Perhaps it represents merely another example of individual variation. With the exception of No. 13 the sum of the principal anions, chloride and CO_2, gives a value which is fairly constant for each alligator throughout the entire experiment. The slight variations in plasma sodium levels are within the experimental error of our method of determination since we could not spare the plasma for duplicate or triplicate analyses on each blood specimen. Although attempts were made to determine the pH of the urine every time blood was withdrawn from an alligator about one-third of the time the urine volume was inadequate for testing. No significant pH changes over the fasting levels were noted in the specimens which were analysed.

In all, 10 experiments were conducted in the months of January, February, April, May, June, August, September, and November to determine the nature and magnitude of the "alkaline tide." These experiments included a total of 360 individual chloride analyses done on blood specimens taken at varying numbers of hours after feeding. In spite of the number of experiments the exact effect of season on the rate of production of HCl is still in doubt. The greatest response observed following feeding occurred in August and September, a fact which may or may not be significant. It is a disappointment to note that the variation in response from animal to animal and indeed in the same animal at different times is very great, and, although the direction of these plasma changes is always the same, the degree of change and the exact time when the lowest plasma chloride level is reached is almost impossible to predict. Technical difficulties have prevented adequate study of the quantity of hydrochloric acid and volume of the gastric contents, although pH values as low as 2 were observed.

Evidence for the fact that all of the alli-

TABLE II.
Effect of Changes in Plasma pH on Plasma Ca and P. (Avg of 6 animals).

	Cl meq/l	CO_2 mM/l	pH	Ca mg%	P mg%
26 hr after feeding	88	31.4	7.45	11.3	3.53
Fasting	109	22.7	7.28	11.5	3.53

TABLE III.
Individual Variations in Fasting Blood pH.

	Time		
All. No.	10:00 A.M.	11:00 A.M.	1:00 P.M.
5	7.18	7.26	7.07
6	7.13	7.36	7.20
7	7.26	7.42	7.47

gators which were used for this study do respond to feeding may be deduced from an examination of the following figures which represent the lowest plasma chloride levels ever found in each of the 11 animals. These values are 40, 75, 46, 45, 57, 27, 50, 13, 7, and 41 milliequivalents per liter. It is possible and even probable that had we known the exact time of maximum response to feeding of all of these experimental animals we might have found chloride values even lower than these reported here. The lowest fasting level ever found was 83 and the average of 98 fasting chloride determinations was 111 milliequivalents per liter.

The high pH levels often observed made it desirable to determine the effect of these drastic pH changes on the plasma calcium and phosphorus contents. Several experiments were conducted and the results of one of these are to be found in Table II. We could find no effect of changes in pH, chloride or CO_2 levels on plasma calcium and phosphorus.

The least reliable information on the acid-base balance of the alligator is furnished by determination of blood pH. Although it is true that the pH of the blood does rise following feeding the amount of this increase is variable. It is possible that the extremely irregular rate of breathing causes wide variations in the amount of free H_2CO_3 in the plasma which results in fluctuations in the $H_2CO_3/NaHCO_3$ buffer ratio. Table III illustrates the variations in the pH change in 3 fasting alligators under conditions in which the environment and temperature were kept constant.

The composition of the ashes of several 1-year-old alligators with respect to some of the more important electrolytes involved in acid-base regulation is presented in Table IV. On the assumption that all of the chlorides assume an extracellular position in the body (an assumption which is not necessarily valid) the total extra-cellular fluids may be calculated from the chloride content. The volumes of extra-cellular fluids calculated in this manner vary from about 19 to about 32% of the body weight. The total sodium and potassium contents showed far less deviation than the chlorides which suggested that chloride had been lost in the ashing of No. 5 and No. 14. Since all of the ashings were done at the same temperature (460°C) and in the same oven it seemed plausible to suspect that the excessive chloride loss may have been due to the volatilization of free gastric HCl. No information is available on the plasma chloride or CO_2 contents of alligators 1, 2, 5 and 48 just prior to ashing. The plasma chloride content of 14, which was fed 18 hours before ashing, was 51 m.eq./l and the plasma chloride content of 23, an animal in the fasting state, was 90 m.eq./l. This indicates that some HCl may have been lost in the ashing although the evidence is by no means conclusive. Further studies are necessary to clarify this point.

Experiments with histamine on the "alkaline tide" met with little success. Amounts varying from 0.01 mg/kilo to 10 mg/kilo were given intramuscularly to several different alligators and blood samples were taken at intervals of from as early as 5 minutes to as late as 27 hours after the injection. The lower doses had little apparent effect while with one possible exception the higher doses produced an acidemia instead of an alkalemia. It is possible that the increased acidity of the blood was due to anoxia caused by respiratory depression since the animals appeared to be semi-moribund for a large part of the experiment. It is still possible that our dosage was wrong and that a study

TABLE IV.
Composition of the Alligator.

All. No.	Wet wt, g	Dry wt, g	H_2O, %	Ash wt, g	Cl m.eq.	Na m.eq.	K m.eq.
1	111.0	25.7	76.8	4.658	3.93	5.35	4.69
2	92.7	22.7	77.6	3.660	3.23	4.45	3.88
5	177.5	43.8	75.0	6.803	3.70	7.07	8.29
48	85.8	20.8	75.8	3.488	3.02	3.52	3.53
14	140.0	31.2	77.7	5.547	3.58	5.80	6.16
23	93.0	18.7	79.9	3.900	3.08	4.03	3.72

of several more animals may yet reveal some effect of histamine.

Summary. The alkaline tide of the alligator has been studied. Although the plasma electrolyte changes were qualitatively similar to those reported for other animals the magnitude of these changes was much greater. Some plasma chloride levels as low as 15% of the fasting level were found and some plasma bicarbonates were elevated 5-fold. Although the average fasting blood pH is about 7.3, pH values of over 8 have been observed at the height of the "alkaline tide." Plasma sodium concentrations were unaffected by feeding. No correlation was found between pH and plasma calcium content.

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Destruction of Pancreatic Acinar Tissue by DL-Ethionine.* (18073)

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The liver's dependence upon dietary factors for its structural and functional integrity is now fully accepted. That such factors also influence the exocrine portion of the pancreas is becoming increasingly clear. Chernick *et al.* found that the feeding of raw soy bean meal induced pancreatic hypertrophy and increased proteolytic activity in the chick(1). Grossman *et al.* reported atrophic pancreases in rats fed a low protein-high fat diet(2) whereas chronic cystic fibrosis was observed, by the Gillmans, in rats fed mealie pap and sour milk(3). In this laboratory, pancreatitis, in which the interstitial tissue appeared to be primarily involved, was induced in dogs by feeding them a high fat-low protein diet(4). Interest in these experimental lesions was increased by the recent suggestion of Davies that kwashiorkor, a nutritional disorder widespread in Africa, is essentially of a pancreatic nature(5). Davies' report was quickly followed by two letters in *Lancet* which brought to light further instances, in man, of pancreatic dysfunction accompanying severe nutritional deficiency(6,7).

We have recently observed that the feeding of a high fat-low protein diet in rats resulted in zymogen degranulation of the acinar tissue of the pancreas, and that methionine prevented its occurrence. This protective action of methionine led us to study the effects of DL-ethionine upon the pancreas. It is shown here that the administration of this analogue of methionine destroys the acinar portion of

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† Atomic Energy Commission Fellow.

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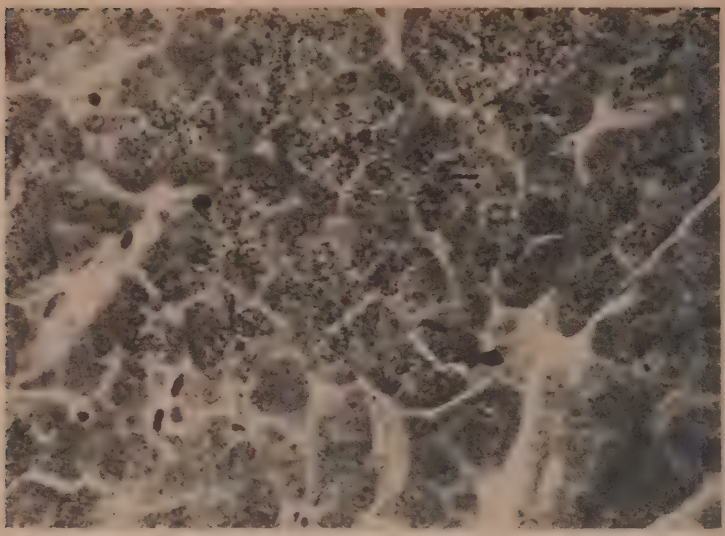

FIG. 1.

Normal rat pancreas. The apical portion of the acinar cells are filled with zymogen granules (black in photograph). Note the cytoplasmic basophilia at the base of the cells. All glands were fixed by intra-arterial injection of Zenker-formol; sectioned at 4 microns. Masson: ×500.

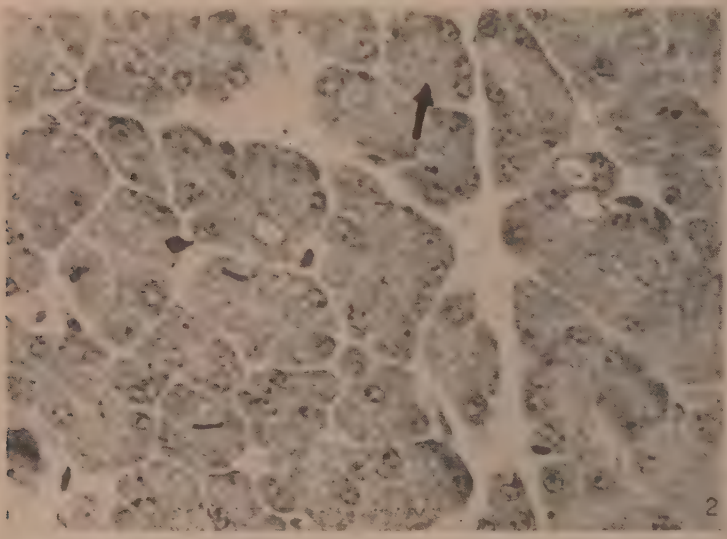

FIG. 2.

Pancreas from rat sacrificed 12 hours after injection of 50 mg DL-ethionine. Note almost complete absence of both zymogen granules and cytoplasmic basophilia. Acinar cells at tip of arrow still are sparsely granulated. Masson: ×500.

the gland. The islet tissue appears histologically normal.

Experimental. Male and female rats, weighing 200-250 g, were injected intraperitoneally, daily, with 50 or 100 mg of DL-ethionine for 1 to 14 days. The rats had ac-

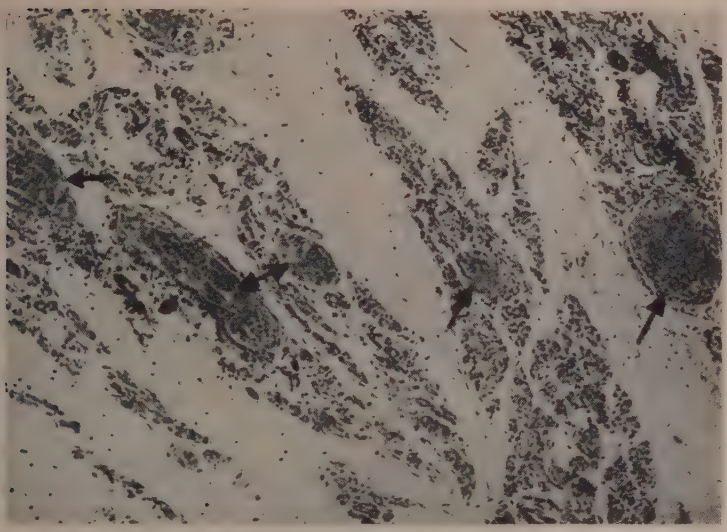

FIG. 3.
Pancreas from rat which received 100 mg DL-ethionine per day for 14 days. Arrows point to islets embedded in the atrophic, fibrotic gland. At this time only a few scattered identifiable acinar cells were observed. Masson: ×100.

cess to food and water until they were sacrificed.

Cytological changes were observed in the pancreas as early as 12 hours after a single injection of 50 mg of DL-ethionine. The acinar cells showed loss of zymogen granules and of cytoplasmic basophilia (Fig. 2). In 24 to 48 hours, the acinar tissue was almost completely degranulated and had lost its cytoplasmic basophilia. The nuclei of these cells exhibited coarser, deeper staining nuclear membranes, and there was clumping of the chromatin material. Distortion of the typical acinar architecture was evident at this time. Dilation of the ducts was prominent; many contained an eosinophilic granular material. No histological alterations were observed in the islets of Langerhans.

Marked pancreatic atrophy and fat necrosis were prominent in rats that had been injected daily with ethionine for one week. Microscopic examination revealed nuclear pyknosis and fragmentation, hydropic degeneration, and cellular disintegration. The tissue was edematous, and a leucocytic infiltration was evident. Exocrine tissue had been gradually replaced by newly laid down connective tissue.

After 2 weeks of ethionine injections, only occasional, scattered single exocrine cells could be identified in the fibrous stroma; some of these contained zymogen-like granules. The dilated ductules appeared unaffected, and the islets still showed no pathological alterations (Fig. 3).

Farber and coworkers(8-10) have shown that the injection of ethionine induces fatty livers in the rat and that this response is much more prominent in the female than in the male. This observation has been confirmed by Jensen in this laboratory (unpublished data). Interestingly enough, the destruction of acinar tissues after injection of ethionine was widespread in both sexes.

The rats suffered marked loss in body weight as a result of the injections. Diarrhea was observed 5-6 days after the start of treatment. An increase in the histologically demonstrable lipid was found in the liver as

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10. Koch-Weser, D., Farber, E., Szanto, P. B., and Popper, H., *Fed. Proc.*, 1950, v9, 336.

early as 6 to 8 hours after injection, and rose to a maximum in about 48 hours. At that time, the entire liver parenchyma was involved, all hepatic cells being distended with lipid globules. A progressive loss of liver lipids was noted after that time despite continued ethionine injections.

One group of rats was fed the stock diet to which had been added 0.1 per cent DL-ethionine. Although the food intake varied widely (0-14 g per day, average of 5 g per day), the effect on the pancreas was no different from that observed when the ethionine was injected.

Comment. It has been known since 1924 that fatty livers developed slowly in depancreatized dogs kept alive with insulin, and that the addition of raw pancreas prevents this fatty change(11). The finding that a similar type of fatty liver occurs in dogs subjected to complete ligation of their pancreatic ducts has led to the view that the release of pancreatic juice into the intestinal tract is necessary for inhibiting the deposit of excessive amounts of fat in the liver. This led us to question whether the fatty liver found in the DL-ethionine-treated rats resulted from destruction of their pancreatic acinar tissue. However, the rapid onset of this fatty change would appear to cast doubt on the possibility that it results from loss of the external secretion of the pancreas.

The view that ethionine inhibits protein synthesis has been expressed by Simpson *et al.* (12). The rapid depletion of zymogen granules and the loss of cytoplasmic basophilia (presumed to reflect the nucleoprotein content of the cell) might be construed to support this view(13-18). It should be stressed, however, that a direct toxic action of the administered ethionine (or a derivative) upon the acinar tissue is not ruled out.

Summary. 1. The effects of daily administration of 50 or 100 mg of DL-ethionine on the rat pancreas is described.

2. Degenerative changes in the acinar tissue occurred as early as 12 hours after a single injection of 50 mg DL-ethionine.

3. Daily injections of 50 or 100 mg in nonfasted rats, for 2 weeks, resulted in almost complete obliteration of the acinar tissue accompanied by fibrous proliferation.

4. No histological evidence of islet damage was observed with these dosages.

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Passive Transfer of Atopic Hypersensitiveness in Man by Means of Leucocytes.* (18074)

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Lawrence(1) has recently demonstrated, in man, the local passive transfer of the delayed type of cutaneous hypersensitiveness to tuberculin by the intracutaneous injection, into normal recipients, of viable leucocytes obtained from the blood of tuberculin-sensitive

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† Dazian Foundation for Medical Research Fellow.

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donors. Chase(2) had previously succeeded in passively sensitizing normal guinea pigs to the delayed type of sensitivity to tuberculin and to chemicals(3) by the injection of leucocytes obtained from peritoneal exudates of sensitized animals. In the present communication, the writers report the local passive transfer of the immediate whealing type of cutaneous reaction which is mediated by the atopic reagin, employing as the skin-sensitizing agent the viable washed leucocytes obtained from the blood of atopic skin-sensitive patients. The experimental method represents a variation from the passive transfer technic originally described by Prausnitz and Küstner(4) in that the transfer was effected by the donor's leucocytes instead of his blood serum.

Methods. Viable leucocytes were obtained from the heparinized venous bloods of allergic patients according to the technic of Minor and Burnett(5) which employs bovine fibrinogen, Fraction I (Armour), to hasten the sedimentation of the erythrocytes. The white blood cells which remained suspended in the plasma were isolated by centrifugation and washed two or four times in Tyrode solution in Machlett capillary-tip tubes, according to the method described by Lawrence(1). Microscopic examination of the cellular sediment after 2 washings revealed a mixture of leucocytes and erythrocytes in which the viable white cells predominated. The average yield from each 10 ml of donor's blood was 0.028 ml of cellular sediment. This sediment, resuspended in 12 times its volume of Tyrode solution, was used for passive local sensitization. Rigid precautions for the maintenance of sterility were enforced throughout the entire procedure.

Sedimented erythrocytes, obtained from the same blood samples which provided the leucocytes, were washed twice and resuspended in Tyrode solution by the same technic employed for the white blood cells. This erythrocytic suspension was also used for attempts at passive local sensitization.

To determine whether plasma, in the dilution which remained in the white cell suspension, could produce passive local sensitization, 0.03 ml of cell-free plasma was treated as though it were cell sediment. It was "washed" (diluted) with 2.0 ml of Tyrode solution and centrifuged in a Machlett tube. With 0.03 ml of "sediment" of this mixture, the "washing" (dilution) process was repeated. Finally, 0.03 ml of the "sediment" was diluted with 12 times its volume of Tyrode solution and used for attempts at passive sensitization.

Sensitized cutaneous sites were also prepared with 1:5 dilutions of the supernate obtained when the plasma was centrifuged to remove the leucocytes.

The passive transfer technic employed in these experiments followed the principles outlined by Walzer and Bowman(6) for indirect testing in atopic hypersensitiveness. Normal nonatopic adults who showed completely negative reactions to skin tests with the concentrated allergens to be employed in the studies were chosen as recipients.

Sites were prepared on the outer aspects of the recipient's arms by the intracutaneous injection of 0.05 ml of the sensitizing substances. At the sites injected with leucocytes, induration and erythema developed, which usually lasted for from three to seven days. Persistence of the inflammation beyond this time rendered the sites unfit for testing. The injection of the erythrocyte suspensions and the serum preparations produced little or no inflammation.

The allergenic extracts used for testing were those routinely employed for diagnosis and treatment in the Allergy Clinic. They were prepared according to the technic of Coca and Milford(7) and standardized on the basis of their total nitrogen content. The ragweed, timothy, and English plantain pollen extracts were made in January, 1950. Because the

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TABLE I.
Passive Transfer of Sensitivity to Ragweed Produced with Leucocytes of H. R.

Recipient	Date of sensitization	Allergen tested (mg N/ml)		Reactions obtained on cutaneous sites prepared with: H. R. leucocytes washed 2×	H. R. leucocytes washed 4×	Normal leucocytes washed 2×	H. R. plasma dilution 1:5
P	4/ 7/50	Ragweed	0.1	+	±		++
P†	4/25/50	"	0.1	+	±		
I	4/27/50	"	0.1	+	±		
D	5/ 1/50	"	0.1	+			+±
P	5/ 6/50	Timothy	0.1	0			
K	5/17/50	Ragweed	0.1	+			
		Timothy	0.1				±
PR	6/19/50	Ragweed	0.1*	+		0	
		"	0.2	±			
P	6/19/50	"	0.2	+			
		"	0.1*	±		0	
		Rabbit epithelium	0.05	0			
		Timothy	0.1*	0			
W	6/19/50	Ragweed	0.1*			0	
		"	0.2			0	

* Freshly prepared extracts.

† Sites on this recipient prepared with twice-washed erythrocytes and with a serum dilution control reacted negatively to tests with ragweed 0.1.

Reactions to tests with above allergens on normal cutaneous sites of recipients were uniformly negative.

potency of the pollen extracts gradually diminished with age, fresh extracts of timothy and ragweed pollens were prepared in June, 1950.

The cutaneous sites on the recipients were tested for hypersensitiveness seven days after their preparation. About 0.02 ml of the allergenic extract was injected into the site and into adjacent normal skin as a control. Positive reactions at sites prepared with leucocytes tended to develop slowly and reached their peak in from 20 to 30 minutes. The larger positive reactions at the leucocytic sites consisted of a wheal and erythema similar to positive reactions on plasma-sensitized sites. Palpation of the reactions at leucocytic sites, however, often revealed a deep-seated edema, which tended to outlast reactions at the plasma-sensitized sites. It was, therefore, necessary to evaluate reactions by palpation as well as by inspection. Pruritus, if present at all, was of a mild degree.

Since the tests with allergens on normal skin produced uniformly negative reactions as a result of the preliminary screening of the recipients, the results of passive transfer tests were evaluated solely on the basis of the reactions at the sensitized sites after twenty minutes. They were graded as follows:

0 = Negative—Site unchanged; 0± = Negative plus—Barely perceptible edema; ± = Slight minus positive—Definite, but small amount of edema, with faint erythema; + = Slight positive—Wheal, at least 0.5 cm in diameter, with surrounding erythema; ++ = Moderate positive—Wheal, approximately 1.0 cm in diameter, with surrounding erythema; +++ = Marked positive—Wheal, larger than 1.0 cm in diameter, with surrounding erythema.

The viable leucocytes used for passive sensitization were obtained from patients with bronchial asthma or hay fever who were attending the Allergy Clinic. They varied widely in the degree of their clinical and cutaneous sensitivities as well as in their serum titers for atopic reagins. None were sensitive to an unusually pronounced degree. Leucocytic suspensions from normal, nonatopic subjects were also used as controls.

Because of convenience, H.R., a hospital interne, aged 27, was used repeatedly as a source of leucocytes (Table I). He had been troubled with hay fever caused by ragweed pollen for two seasons. Symptoms rarely occurred during the grass (timothy) season. He

TABLE II.
Summary of Passive Transfer Studies with Leucocytes from Allergic Patients.

Donor; Diagnosis; Skin reactions	Recipient	Date	Allergen tested (mg N/ml)	Reactions obtained on cutaneous sites prepared with: Leucocytes washed 2×	Leucocytes washed 4×	Serum control	Plasma: Dilution	Plasma: Result
T.C. male, 26 yr	P	4/ 6/50	Rabbit epith. 0.05	+			1:5	++
Asthma	G	4/17/50.	" " 0.05	±	0±			
Rabbit epith. 0.001 ++±	D	4/22/50	" " 0.05	±	0			
S.F. female, 30 yr	W	3/13/50	Ragweed 0.1	+		0	None	+++
Ragweed hay fever	K	5/29/50	" 0.1	±				
" 0.001 +			" 0.2	±				
" 0.01 +++			Timothy 0.1	0±				
Timothy 0.1 ++			Saline	0				
M.S. female, 45 yr	D	4/10/50	Ragweed 0.1	±		0	1:5	++
Ragweed hay fever	PR		" 0.2	+		0		
" 0.0001 ++			Plantain 0.1	0				
" 0.001 ++±			Timothy 0.1	0				
" 0.01 +++			Cat epith. 0.01	0				
Timothy 0.1 ++±								
Plantain 0.1 ++								
Cat epith. 0.01 0								
S.E. male, 27 yr	P*	4/ 4/50	Ragweed 0.1	±	0		1:5	++
Ragweed hay fever								
" 0.001 +								
" 0.01 +++								
V.P. male, 11 yr	T	3/28/50	Ragweed 0.1	0	0			
Asthma			" 0.2	0	0			
Ragweed 0.001 +	P		" 0.01				None	++±
" 0.01 +++								

* Sites on this recipient prepared with twice-washed erythrocytes reacted negatively to tests with ragweed 0.1.
Reactions to tests with above allergens on normal cutaneous sites of recipients were uniformly negative.

reacted to intracutaneous tests with pollen extracts as follows:

Ragweed	0.0001	+
"	0.001	++±
"	0.005	+++
Timothy	0.1	+
Plantain	0.1	+±

Titration of the reaginic content of his blood serum by the serum dilution method of Coca and Grove(8) with an extract containing 0.01 mg N/ml showed a titer of 1:64 to ragweed and 1:4 to timothy.

Results. Cutaneous sites on five different recipients prepared with the leucocytes of ragweed-sensitive donor, H.R., responded regularly with positive reactions to tests with ragweed in every instance (Table I). Two tests on these sites with timothy pollen and one with rabbit epithelium gave negative reactions. Sites prepared with a suspension of twice-washed erythrocytes and with the serum dilution control of H.R. did not react to tests with ragweed. Leucocytes from a nonsensitive donor failed to sensitize the recipient's skin to ragweed. Washing the leucocytes of H.R. 4 times instead of twice diminished but did not eliminate their specific skin-sensitizing property. The weak reaction to timothy pollen obtained on the plasma site could not be elicited on leucocytic sites.

In studies with leucocytes obtained from 5 other atopic patients, positive transfers were obtained in 4 (Table II). With the cells of T.C., local cutaneous sensitivity to rabbit epithelium was induced in 3 recipients. Skin-sensitizing antibodies to ragweed were transferred with leucocytes of 3 of the remaining four patients. All sites prepared with suspensions of erythrocytes, with serum dilution controls, and with viable leucocytes from nonsensitive donors gave negative reactions to tests with ragweed. The relatively weak sensitivities to timothy and plantain in patients S.F. and M.S. were not transferred with their leucocytes.

Failure to transfer sensitivity to ragweed occurred with the leucocytes of patient, V.P., an 11-year-old male, with a positive skin reaction to ragweed, but with no clinical history of pollen sensitivity. This patient's plasma readily transferred ragweed sensitivity.

Discussion. The positive reactions elicited in these experiments on sites sensitized with leucocytes were specific and definite, but no reaction greater than one-plus was obtained. The fact that the leucocytes were provided by donors with sensitivities of only average intensity may account, in part, for the relatively weak transfers. The inflammatory reaction which followed the sensitizing injection at the leucocytic sites was undoubtedly an important factor in reducing the intensity of the response of these sites to subsequent tests with allergens. Tests with weak solutions of histamine on leucocyte-prepared sites showed a diminished response as compared to that obtained on normal adjacent skin similarly tested at the same time. Moreover, experience obtained in the testing of sites sensitized with serum has revealed that the specific as well as the nonspecific responsiveness of passively sensitized sites is reduced by the inflammation which results from the sensitizing injection(9).

Conclusions. 1. The intracutaneous injection of viable leucocytes obtained from the bloods of atopic subjects who respond with immediate whealing reactions to skin tests with common allergens may specifically and locally sensitize the skin of normal recipients.

2. Red blood cells obtained from the same donors lack this skin-sensitizing property.

The authors gratefully acknowledge the technical assistance of Sonia Stroyman.

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Adrenal Cortex and Lipid Metabolism: Effects of Cortisone and Adrenocorticotropin (ACTH) on Serum Lipids in Man.* (18075)

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It has now been established that cortisone and ACTH affect the metabolism of protein and carbohydrate in man. These effects are manifested by an increased urinary excretion of nitrogen, as well as hyperglycemia and glycosuria(1). It is the purpose of this report to present evidence that lipid metabolism is also affected.

From clinical observation it has been known that patients with hyperfunction of the adrenal cortex (Cushing's syndrome) have hypercholesterolemia and an abnormal distribution of body fat, whereas individuals suffering from adrenal insufficiency (Addison's disease) tend to a low serum cholesterol and a loss of body fat(2). In the animal experiment fatty infiltration of the liver produced by pancreatectomy has been prevented by adrenalectomy(3); marked hypercholesterolemia was observed in rabbits after adrenal homotransplantation or injection of adrenal cortical extracts(4); and loss of body fat in the adrenalectomized rat was prevented by the administration of cortisone(5).

The availability of ACTH and cortisone has now made it possible to study further the physiologic effects of these hormones. The following results were obtained in 15 persons with various diseases who were given cortisone in doses varying from 25 to 200 mg a day. In Table I the basic disease, the duration of hormone administration, the dosage, and the changes of total cholesterol, phospholipids, and neutral fat levels of the serum are presented. The determination of total and esterified cholesterol was done by the method of Schoenheimer and Sperry, lipid phosphorus by the Sperry modification of the Fiske-Subbarow method (phospholipid equals lipid P times 25), total lipids were determined by the Bloor method, and neutral fat was calculated by subtracting the figures for total cholesterol and phospholipids from the figure for total fat.

It is evident from Table I that there was an average increase of 15% in the total serum cholesterol of the 15 patients who received cortisone. The phospholipids increased an average 26% with 14 of the 15 showing this change. The neutral fat was calculated in 12 instances, and in each case there was a decrease in this fraction; the average drop was 51%. By using the t-distribution for statistical evaluation, a probability of .032 was obtained for the change in phospholipids, and of .005 for the change in neutral fat.

The fluctuations of serum lipids under cortisone occurred regardless of the clinical course of the patient or the underlying disease. They occurred in patients 4, 6, and 12 although no symptomatic improvement was observed. In patient 11, who died, and in patient 14, who failed to improve, the serum phospholipids rose, but the level of serum cholesterol fell. The described changes occurred in patients 2 and 6 even while they were maintained on a fat-free, cholesterol-free, salt-free diet (Kempner regimen). In 4 patients (1, 2, 3, and 6) studies of the serum lipids were continued after cortisone was stopped, and in each instance there was a reversal of the previous trend, *i.e.*, the cholesterol and phospholipids fell, and the neutral fat rose, so that the values eventually approached those of the control periods.

In contrast to the effects of cortisone,

* This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service.

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TABLE I. Effects of Cortisone.

Pt.	Disease	Dose, mg	Days	Cholesterol*			Phospholipids*			Neutral fat*		
				Before	After	% change	Before	After	% change	Before	After	% change
1	Disseminated lupus	150	10	192	293	+53	240	398	+66	712	169	—77
2	,, ,,	150	10	208	284	+35	240	322	+34	404	216	—47
3	Polyarteritis nodosa	100–150	12	167	218	+23	157	279	+78	356	149	—58
4	Scleroderma	100–150	19	177	210	+19	185	248	+34	273	58	—79
5	Disseminated lupus	75–150	26	167	215	+22	166	260	+57	277	109	—61
6	Chronic nephritis	38–150	9	271	300	+11	339	432	+27	680	506	—26
7	Acute leukemia	100–200	8	163	173	+ 6	179	176	— 2	208	144	—31
8	Hodgkin's disease	60	21	183	167	— 8	227	230	+ 1	—	—	—
9	Disseminated lupus	100–150	12	211	248	+17	271	322	+19	241	120	—50
10	Rheumatoid arthritis	25–200	23	155	200	+29	217	268	+22	301	287	— 5
11	Disseminated lupus	150–200	19	212	205	— 3	232	256	+11	191	139	—27
12	Chronic leukemia	150	6	153	186	+22	188	238	+27	—	—	—
13	Dermatitis	100–200	6	500	510	+ 2	421	485	+15	—	—	—
14	Chronic leukemia	100–150	9	131	100	—22	205	252	+23	421	81	—81
15	Dermatomyositis	25–150	9	239	286	+20	331	365	+10	270	154	—43
			Avg	209	240	+15	240	302	+26	361	178	—51

* In mg per 100 ml of serum.

TABLE II.
Effects of ACTH.

Pt.	Disease	Dose, mg	Days	Cholesterol*			Phospholipids*			Neutral fat*		
				Before	After	% change	Before	After	% change	Before	After	% change
101	Polyarteritis nodosa	5–100	28	194	270	+39	280	304	+ 9	154	202	+31
102	Scleroderma	10– 75	17	210	198	— 4	232	244	+ 5	—	—	—
103	Disseminated lupus	25– 75	28	215	198	— 8	260	222	—15	—	—	—
104	Pemphigus	25– 80	13	161	184	+14	220	223	+ 1	169	73	—55
105	Disseminated lupus	10– 75	34	248	219	—12	322	295	— 8	120	106	—12
106	,, ,,	75–100	25	188	199	+ 6	260	205	—21	139	46	—67
107	,, ,,	100	5	289	278	— 4	349	352	+ 1	352	493	+40
108	,, ,,	50–100	15	132	111	—16	174	285	+63	177	21	—68
109	Hypoproteinemia	100	11	175	156	—11	205	268	+30	—	—	—
110	Disseminated lupus	50–100	11	264	295	+11	285	311	+ 9	341	301	—12
111	Polyarteritis nodosa	50–100	11	174	212	+10	222	310	+40	167	118	—29
112	Disseminated lupus	25– 75	15	246	271	+10	299	350	+17	—	—	—
			Avg	208	216	+ 4	259	281	+ 9	202	170	—16

* In mg per 100 ml of serum.

ACTH produced less pronounced changes in the serum lipids (Table II). A small drop in total serum cholesterol was noted in 6 of 9 patients during the first few days of treatment as originally observed by Conn(6), but this was usually followed by a subsequent moderate rise, and the overall average for the group showed an increase of 4%. There was an average increase of 9% in the phospholipids with 9 of the 12 exhibiting a rise, and an average decrease of 16% in the neutral fat, with 6 of 8 showing this effect. None of the changes which occurred during ACTH therapy were statistically significant.

Of special interest was the fact that the fasting sera of 7 of the 15 cortisone-treated patients and 3 of the 12 ACTH patients became turbid ("lipemic") despite the fact that the level of neutral fat had decreased. This may indicate that the rise in phospholipids and cholesterol was due to the production of a lipid which was less soluble than normal, or that there was a change in the conjugated lipids (possibly lipoproteins).

It is evident, then, that there was a difference between the effects of cortisone and ACTH on the serum lipids. This difference of effects may be explained by the different nature and points of attack of these substances. Cortisone is a cortical hormone of a well-defined structure, while ACTH is an organ extract of the anterior lobe of the pituitary which stimulates the production of several cortical hormones. Evidence is available(1) that the adrenal cortex of man when stimulated by ACTH secretes Compound F rather than cortisone (Compound E). It is also possible that serum cholesterol represents the material for the synthesis of cortical hormones under ACTH stimulation(6), and that prolonged stimulation results in functional exhaustion and atrophy of the adrenal cortex, as well as in the production of antihormones(7).

Summary. This investigation indicates that cortisone produced well-characterized changes of serum lipids in man. The effects of ACTH on the serum lipids were less pronounced.

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Positive Inotropic Action of Ouabain on Rat Ventricle Strips.* (18076)

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Previous work in this laboratory on the metabolism and functional activity of the rat heart(1,2) led us to an investigation of the effect of cardiac glycosides on the force of contraction of rat ventricle muscle. The present paper reports a positive inotropic action of ouabain on the isolated rat ventricle strip, and the effects of various factors upon this response. A literature survey reveals that this is the first conclusive demonstration of a cardiotonic effect of ouabain on rat myocardium.

Method. Adult male rats (225-275 g) were killed by decapitation and a strip prepared from the right ventricle, attached to a semi-isometric optical lever, and mounted according to the method developed in this

* This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

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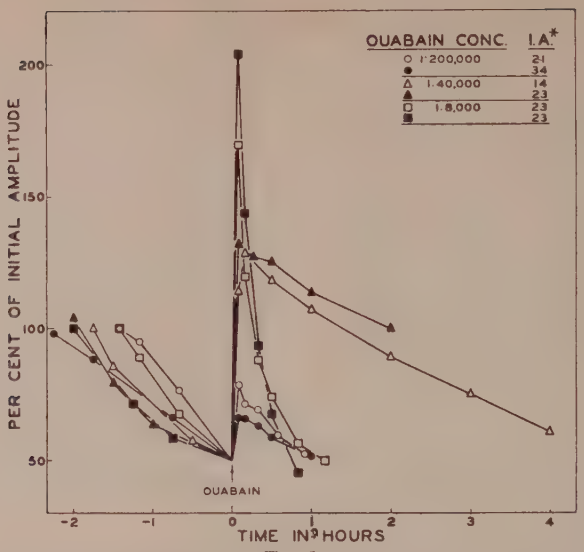

FIG. 1.

Effect of ouabain concentration. * I.A. Initial amplitude of contraction in mm ×65 (after equilibrating 1 hr).

laboratory by Feigen *et al.*(3). The tissue was placed in a medium containing 0.9% NaCl, 0.042% KCl, 0.036% $CaCl_2$, 0.1% glucose, and 0.00105 M phosphate buffer (pH 7.2) at 37.5°C, and gassed with oxygen through a sintered glass plate in the bottom of the vessel. The muscle, with a diastolic tension of 0.75 g, was stimulated at a constant rate of 100 per minute by an Electrodyne stimulator. After an equilibration period of one hour, the solution in the vessel was renewed. The contractions, magnified 65-fold, were recorded photographically every 15 or 30 minutes during the "fatigue" period in a manner similar to that of Cattell and Gold(4,5), who first developed the technic for demonstrating the positive inotropic effect of cardiotonic glycosides on the cat papillary muscle. When the force of contraction had decreased to approximately 50% of the initial value, ouabain was added and a record was taken every 5 to 15 minutes. Only one addition of ouabain was made for each ventricle strip tested.

Results. Effect of ouabain concentration. When ouabain was added to the hypodynamic ventricle strip, there was a very rapid increase in the force of contraction, usually reaching a maximum in 3 to 5 minutes. Typical results of 3 different concentrations are presented in Fig. 1. A fairly uniform response was obtained with each concentration. At 1:40,000 ouabain there was a pronounced positive inotropic effect which remained above the 0 time value (Fig. 1) for more than 4 hours, whereas the force of contraction following 1:8,000 returned to the 0 time value in approximately one hour even though the maximum developed tension was greater. Inasmuch as a concentration of 1:40 million to 1:75 million produces a cardiotonic effect on the cat papillary muscle(4), an effective concentration of 1:40,000 is not unexpected for the rat ventricle preparation, as the toxic dose for a rat is 100 to 1000 times greater (depending on the rate of injection) than for a cat(6-8).

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Investigation of time-response to ouabain. As already mentioned, the maximum effect on the rat ventricle strip occurred within 5 minutes. Yet, when the papillary muscle of the cat's heart was exposed to 1:5 million ouabain, the maximum tension was not reached until 45 and 90 minutes in 2 experiments, thus confirming the observations of Cattell and Gold(4). This time difference could be due (a) to a species variation or (b) to a difference in the part of the heart used. Rat papillary muscles from the left ventricles of 2 rat hearts were tested in the same manner as the right ventricle strip. Although the initial force of contraction of the rat papillary muscle was considerably less than the ventricle strip, the maximum increase in force of contraction following the addition of ouabain (1:40,000) also occurred in approximately 5 minutes. It is probable, therefore, that the difference in time-response to ouabain between the cat papillary muscle preparation and the rat ventricle strip is due to a species difference.

Effect of calcium concentration on ouabain response. The relation between the ouabain response and calcium concentration was investigated, and Fig. 2 shows the response at 3 calcium concentrations. Feigen *et al.*(3) observed that the rat ventricle strip exhibited maximal contraction at a concentration of 0.062% $CaCl_2$ and above or below this amount there was a decrease in activity. With an increase in calcium from 0.012% to 0.036%, we found an increase in the ouabain response, but at a concentration of 0.062% $CaCl_2$ ouabain produced a smaller effect than at the two lower concentrations.

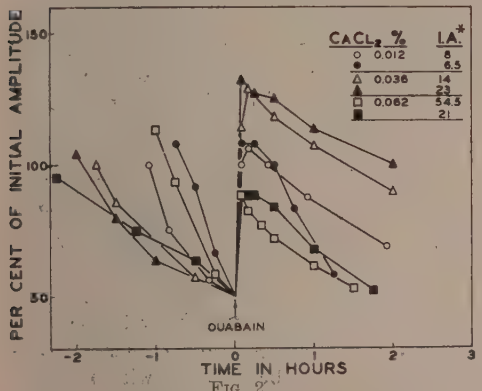

FIG. 2.

Effect of calcium concentration on ouabain response. 1:40,000 ouabain was added at 0 time. * I.A. See footnote under Fig. 1.

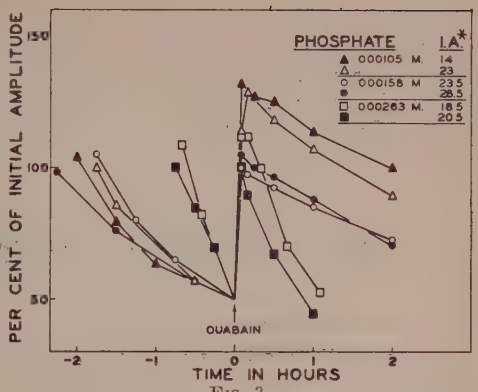

FIG. 3.

Effect of phosphate concentration on ouabain response. 1:40,000 ouabain was added at 0 time. * I.A. See footnote under Fig. 1.

Effect of phosphate concentration on ouabain response. White and Salter(9) found that the cat papillary muscle preparation would contract in bicarbonate buffer without much decrease in force over long periods of time, but in phosphate buffer a rapid decrease in mechanical activity occurred. We have, therefore, investigated the effect of various phosphate concentrations on the ouabain response. Fig. 3 shows that ouabain produced the greatest effect in the presence of a low concentration of phosphate. The highest concentration of phosphate produced the most rapid "fatigue," and even after ouabain this depressant effect of phosphate was apparent.

Effect of initial tension. The amount of diastolic tension on the muscle was varied to determine its effect on the ouabain response. The maximum response to ouabain (1:40,000) at diastolic tensions of 0.25 g and 1.5 g was approximately two-thirds that obtained at a tension of 0.75 g.

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Summary. A positive inotropic effect of ouabain has been demonstrated on the rat ventricle strip. At a given ouabain concentration the magnitude of the response was quite uniform. The effects of variation in the concentration of ouabain, calcium, and phosphate, and in initial tension on the positive inotropic response have been investigated.

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Band-Pass Filter Action of the Cochlea During Nembutal Anesthesia.* (18077)

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In the course of studies of the potentials evoked in the cerebral cortex by auditory stimulation, we have noted changes in the threshold of the electrical response of the cochlea which merit further description.

Experimental. The observations were made in cats anesthetized with nembutal. The auditory stimuli, provided by a variable frequency sine-wave generator (Hewlett-Packard No. 202B) were led into the ear from a headphone via a plastic tube fixed in the external meatus. Recording of the cochlear potential was accomplished through a wick electrode placed at the round window and led through a suitable amplifier to a cathode-ray oscilloscope. The cochlear audiograms were recorded by determining the threshold intensity of stimulus at various frequencies from 100 to 5000 cycles/sec.

Results and discussion. Typical audiograms in 3 cats, A, B, and C, are charted in Fig. 1. The duration of anesthesia, the body temperatures, and the respiratory rates are indicated at the right of the chart.

During nembutal anesthesia the change in the cochlear response consists of a rise in threshold, initially for the higher frequencies, and later for the lower frequencies. Characteristically, there is a relative sparing of the frequency band around 1700 cycles, as recorded in the audiograms of cats B and C. This differential sparing may result in a relatively narrow frequency band with a threshold 6 or more db below any other.

At low intensities of stimulation, therefore, the cochlea may respond only to a narrow frequency band and its functioning may be likened to that of a band-pass filter.

Riesco-MacClure *et al.*(1) have reported antemortem failure of the cochlear response, especially to higher frequencies, in guinea pigs under Dial anesthesia. They considered this failure as possibly due to "cumulative irreversible or slowly reversible effects of prolonged partial anoxia." Our observations with nembutal anesthesia confirm the finding of these authors that the level of anesthesia, *per se,* and the usual fall in body temperature are not necessarily implicated in the depression of the cochlear response. This depression may occur when the level of anesthesia is light as judged by the electrical activity of the cerebral cortex and by the presence of reflex withdrawal responses. An audiogram with normal frequency distribution may be obtained when body temperature has fallen as in cat A, and a depressed cochlear response may be obtained with a similar fall in body temperature, as in the audiogram B_2.

Although the depression of the cochlear

* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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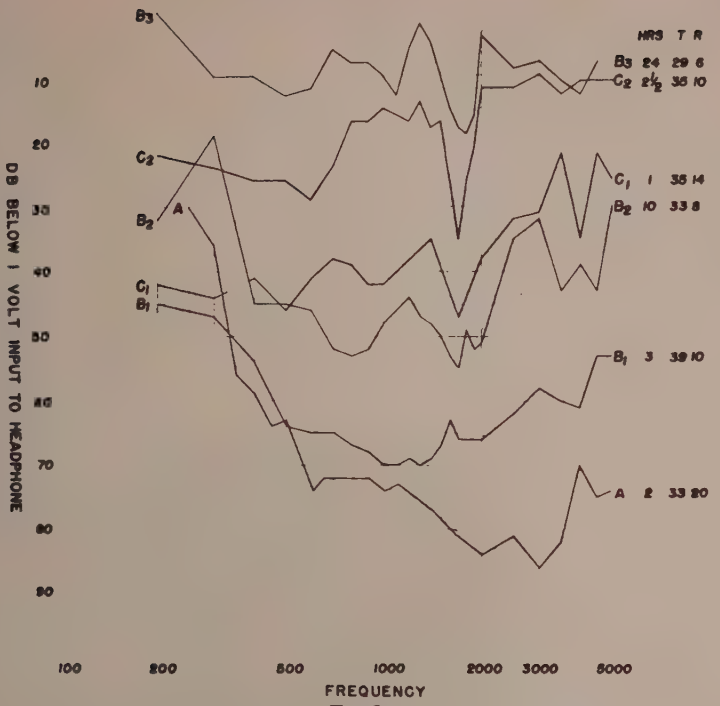

FIG. 1.

Overall cochlear audiograms of Cats A, B, and C, anesthetized with nembutal. Duration of anesthesia, body temperature (°C), and respiratory rate at right.

response is usually seen in long-lasting experiments, it may appear quite early, *i.e.*, within 2½ hours, as in cat C. The most constant finding has been an associated depression of the respiratory rate which may occur early or late in anesthesia. Such a depression of the rate of respiration may support the thesis that anoxia is a causal factor, but its presence does not necessarily indicate that the preparation is moribund. Cat B, for example, at 24 hours had a low body temperature and depressed respiration, but had an electrically active cerebral cortex and active reflex withdrawal responses. An animal in this state may go on for many hours giving good cortical responses to auditory stimulation, without other evidence of deterioration.

Summary. In cats under nembutal anesthesia, there is a selective depression of the cochlear response so that with threshold stimulations it functions as a band-pass filter for frequencies around 1700 cycles/sec. This depression does not appear to be due to the anesthetic, *per se*, nor to fall in body temperature, but occurs in association with depression of the respiratory rate.

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Therapeutic Effect of Aureomycin in Pernicious Anemia.* (18078)

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One of the earliest theories of the pathogenesis of Addisonian pernicious anemia was that of Hunter(1). He postulated as a primary factor the presence of a toxin derived chiefly from the flora of the gastrointestinal tract and occurring in individuals constitutionally predisposed. After the demonstration of the beneficial effects of liver orally(2), liver extract parenterally(3), and the publication of Castle's work on the extrinsic and intrinsic factors(4), the maturation arrest hypothesis was accepted almost universally. Dock(5) challenged this point of view and reviewed the evidence for a hemolytic pathogenetic mechanism. Dobriner and Barker(6) showed that the bilirubin and porphyrin changes of pernicious anemia responding to liver were identical with those of hemolytic anemia after splenectomy, thus reviving the hemolytic theory of pernicious anemia. Whether or not the bacterial flora in the gastrointestinal tract is in any way implicated in the pathogenesis of pernicious anemia might be indicated by the effect of powerful antibiotics given orally to these cases.

It was reported by Stokstad and Jukes(6a) that aureomycin would produce a growth response in chicks on diets deficient in vitamin B_{12}. They suggested that aureomycin might suppress the *E. coli* population and permit the growth of other organisms which synthesize more vitamin B_{12}.

Materials and methods. We have used aureomycin† in 5 cases of Addisonian pernicious anemia in relapse and in one case of nutritional macrocytic anemia. Ages ranged from 55 to 76 years. All marrows were megaloblastic. Complete blood counts and hematocrits were done 3 times a week, the red blood cell counts being done in duplicate. Reticulocyte counts were performed daily. The therapy of each patient will be presented in detail below.

Results. The data are recorded in Table I. In those patients who were transfused before onset of therapy, the initial red blood cell counts recorded are those after transfusion. The first patient (Case No. 1) with Addisonian pernicious anemia was treated with 2 g of aureomycin and 2 g of streptomycin orally each day for 32 days (Fig. 1). This resulted in a delayed reticulocyte peak of 9.6% with a slow concomitant rise in blood values. Bacteriological studies of the stools revealed a gradual decrease in all bacterial growth until the end of the second week of therapy when unidentified yeasts were found in great numbers with almost complete absence of other organisms. The yeasts persisted until the 25th day when they were replaced by almost a pure culture of *Pseudomonas aerogenosa*. In this experiment a good clinical response and a definite, though submaximal, hematological response was elicited in a patient with Addisonian pernicious anemia by the use of oral antibiotics alone. The traces of vitamin

* Paper presented at the annual meeting of the American Federation for Clinical Research at Atlantic City, N.J., on May 2, 1950.

The expenses of this investigation were defrayed by a Lederle grant.

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† The aureomycin used in each of the cases except the first was supplied to us by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y. Their microbiological assay of this lot revealed the presence of 0.17 μg of vitamin B_{12} per gram of aureomycin.

TABLE I.

Case No.	1		2		3		4		5	
Day of R_x	Retic. %	RBC mil./cmm.	Retic. %	RBC mil./cmm.	Retic. %	RBC mil./cmm.	Retic. %	RBC mil./cmm.	Retic. %	RBC mil./cmm.
0	0.4	2.10	1.8	2.71	1.7	1.54	1.5	1.60	1.0	1.68
4	0.2		3.9	2.68	1.5	1.37	6.5	1.70	3.3	1.71
8	0.8	2.89	4.2	2.72	0.9	1.33	1.2	1.73	6.9	2.01
12	1.4		2.8	2.91	1.4	1.24	1.7	1.69	4.7	1.83
16	1.6	2.66	1.9	2.90	2.1	1.18	3.3	1.82	2.3	2.30
20	5.2		2.3	2.81	3.6	1.58	3.6	1.68	1.7	2.42
24	9.6	2.82	3.6	3.01	5.8	1.29	5.2	2.10	1.3	2.47
28	6.2		4.6	3.32	12.2	1.81	4.8	2.18	2.2	2.51
32	2.8	3.21	4.6	3.09	4.8	2.29	1.2	2.56	1.2	2.66
36			3.1	3.05	3.6	2.64	3.9	2.28	1.2	3.06
40			4.3	3.32	2.0	2.83	2.4	2.40		
44					0.9	3.01	3.1	2.64		
48					1.1	3.20	2.5	3.05		

Hematological results of aureomycin given to Cases 1, 2, 3, 4 who had Addisonian pernicious anemia, and to Case 5, who had nutritional macrocytic anemia. Therapy was given orally daily as follows:

No. 1—2 g aureomycin and 2 g streptomycin 0-32nd day.
No. 2—3 g aureomycin 0-40th day.
No. 3—(a) 3 γ B_{12} 0-48th day.
(b) 3 g aureomycin 11-48th day.
No. 4—(a) 2 g aureomycin 0-28th day.
(b) Meat-free diet 0-12th day.
(c) 200 g meat daily 13-28th day.
No. 5—(a) Meat-free diet and 2 g aureomycin 0-36th day.
(b) 3 γ B_{12} 13-36th day.

B_{12} that may have contaminated the aureomycin and streptomycin used here could not possibly have been solely responsible for the therapeutic effect. It has been repeatedly demonstrated that vitamin B_{12} is ineffective orally (7-9) in pernicious anemia except in extremely large amounts or with normal gastric juice.

A second patient was given 3 g of aureomycin daily for 40 days, during which time the hematocrit rose from 33% to 43%, with a slower increase of red blood cells. There was an irregular reticulocytosis of 2-5% from the 5th day of therapy throughout the entire course. This is not the conventional response and therefore cannot be interpreted. Clinical improvement was very satisfactory and was characterized by better appetite and loss of fatiguability.

In the first 2 cases the beneficial effects of the antibiotics resulted without the addition of vitamin B_{12} to that present in the normal hospital diet. It seemed possible that aureomycin might be active hematopoietically through its ability to augment utilization or absorption of vitamin B_{12}. In Case No. 3 (Fig. 2) therefore, vitamin B_{12} was administered orally in a dosage of 3 μg a day. During the first 10 day period this was ineffective, but, with the subsequent addition of aureomycin, there was a clinical and hematological response of a greater magnitude than in the preceding cases receiving antibiotics alone.

It was thought that the effect of extrinsic factor in the form of meat might be enhanced by aureomycin in a manner similar to that of extrinsic factor in the form of vitamin B_{12} in the previous experiment. Case No. 4 was given a diet free of meat, eggs, fish and cheese, plus 2 g of aureomycin orally each day. During the first 12 days there was no response. On the 13th day a daily dietary supplement of 200 g of lean meat was started The red blood cells began to rise slowly but steadily

7. Spies, T. D., Suarez, R. N., Lopez, G. G., Milanes, F., Stone, R. E., Toca, R. L., Arambura, T., and Kartus, S., *J. A. M. A.*, 1949, v139, 521.

8. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M., *New England J. Med.*, 1948, v239, 911.

9. Unpublished data of the authors.

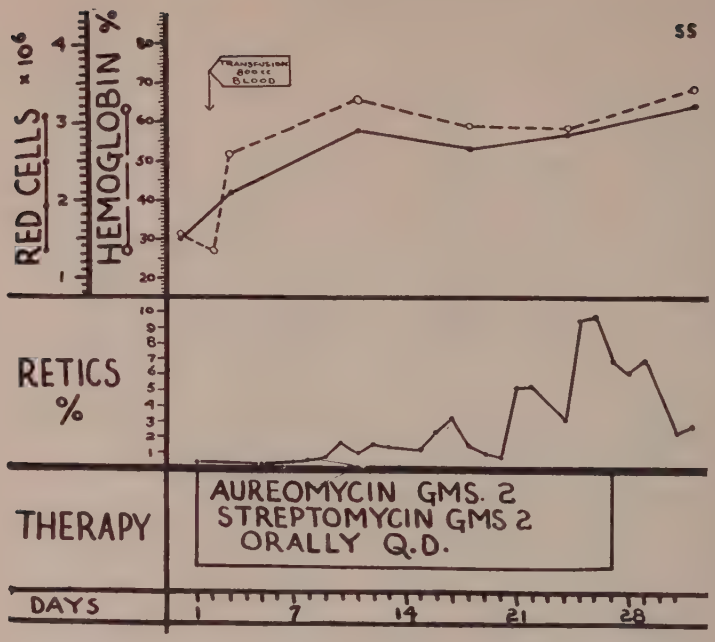

FIG. 1.
Hematological response of Case No. 1 (Addisonian pernicious anemia) to antibiotics alone.

to a value of 3.62 mil per cmm on the 61st day of the experiment. There was an irregular, non-specific type of reticulocytosis which could not be interpreted in relation to therapy. The rise in blood values was associated with an excellent clinical response and a conversion of the bone marrow to a normoblastic type. Since it is well known that a patient with Addisonian pernicious anemia will not respond to administration of meat alone, it was thought that the response here was brought about by the aureomycin.

A study similar to Case No. 4 was performed on a patient (Case No. 5) who had nutritional macrocytic anemia with free HCl in the gastric juice. This patient was given a diet free of meat, fish, eggs, and cheese, and 2 g of aureomycin orally each day. During the first 13 days on aureomycin with no extrinsic factor available to the patient there was an irregular reticulocytosis and a rise in blood elements along with some clinical improvement. The addition of oral vitamin B_{12} for a second period of 18 days seemed to cause no definite secondary response. The later successive parenteral administration of vitamin B_{12} and pteroylglutamic acid (PGA) did not produce another rise in reticulocytes or further rise in red blood cell values.

In all 5 cases the marrow was found to have become normoblastic upon completion of aureomycin therapy. Clinical response was slower than that observed in patients responding to parenteral vitamin B_{12} or liver extract, but was satisfactory. No exacerbations of neurological symptoms were observed. It is interesting that 3 of the 5 cases exhibited an irregular reticulocytosis while on aureomycin. The reason for this is obscure.

Finally, a 6th patient, with Addisonian pernicious anemia, was treated with intravenous aureomycin in order to determine whether the route of administration might be a factor. The initial red blood cell count was 1.68 mil per cmm. The patient was given 600 mg of aureomycin intravenously each day for 20 days, without any clinical or hematological response whatsoever. The re-

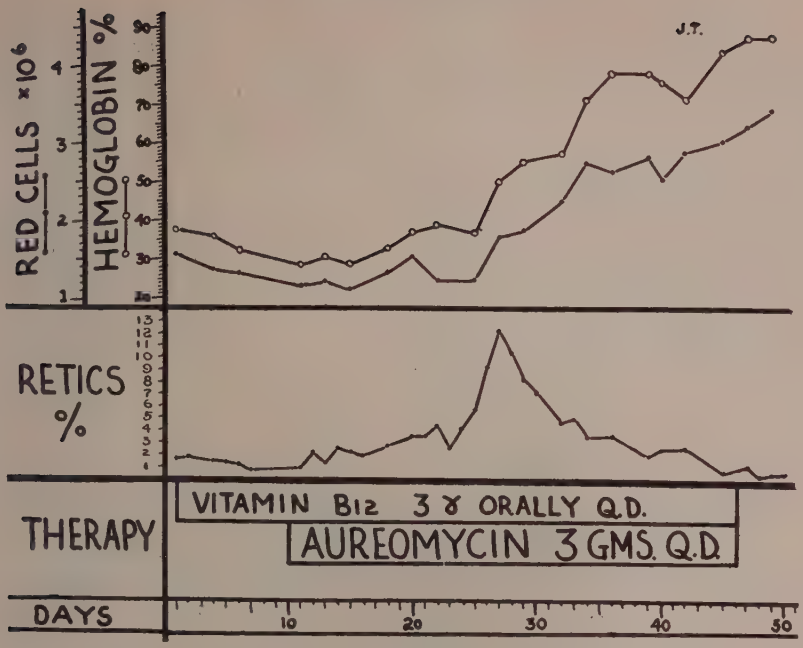

FIG. 2.
Hematological response of Case No. 3 (Addisonian pernicious anemia) to vitamin B_{12} orally only after addition of aureomycin.

ticulocytes never rose above 2.0%. The lack of response was not unexpected because it was thought that the probable site of action of aureomycin was in the intestine, and presumably intravenous administration would not give as high a concentration in the intestine as oral administration. However, further experiments are necessary before definite conclusions can be reached.

Discussion. Although potent therapeutic agents—vitamin B_{12}, PGA, and liver extract—are available for the treatment of pernicious anemia and other related megaloblastic anemias, the pathogenesis of these disorders has not been completely elucidated because the spontaneous remission is unexplained by current theories. If the development of the state of pernicious anemia depends simply upon a failure to absorb vitamin B_{12} due to achylia gastrica, who do so many people with achylia remain immune? The bacterial flora theory had many adherents before the discovery of the beneficial effects of liver. Moensch(10) studied the fecal flora in 33 cases of pernicious anemia and reviewed the pertinent literature. Their results coincided with the finding of others, that there was an unusually large number of viable organisms in the stools of these cases. Particularly numerous were *E. coli* and *Cl. welchii*. Cameron *et al.*(11) demonstrated the production of a macrocytic anemia in rats by the formation of a blind loop in the small intestine. The manner of the development of the anemia in their animals suggested to them "that the decisive event may be a change in the flora of the loop and, possibly, in the remainder of the small intestine. This may lead to the loss of an organism which synthesizes hemopoietic material, or the preponderance of an organism which uses up hemopoietic material, or the presence of an organism which produces an antagonist to hemopoiesis." The anemia

10. Moensch, M. L., Kahn, M. C., and Torrey, J. C., *J. Infect. Dis.*, 1925, v37, 161.

11. Cameron, D. G., Watson, G. M., and Witts, L. J., *Blood*, 1949, v4, 803.

in these rats responded to liver extract and PGA. Unfortunately we have not had an opportunity to study the effect of aureomycin in patients with megaloblastic anemia due to intestinal disease.

The mechanism of action of the aureomycin in our cases remains to be determined. A few of the many possibilities are: 1. The sterilization of the gastrointestinal flora by aureomycin may remove a hemolytic toxin. 2. The lowering of the bacterial population in the gastrointestinal tract may increase the amount of dietary vitamin B_{12} available for the patient. Recent work(12) has demonstrated that *E. coli* has a marked affinity for vitamin B_{12} and can remove it from solution *in vitro*. 3. The lowering of the bacterial flora count by aureomycin may permit the growth of other microorganisms, for example, yeasts, which in turn may produce PGA or other hematopoietically active substances utilizable by the patient. In the only case (Case No. 1) in which studies of the bacterial flora were done we found a great increase in the presence of yeasts. Dearing and Heilman(13) noted a similar increase in yeasts in the stools of the majority of their patients after the use of aureomycin for short periods of time. Smith and Robinson(14) reported that large doses of streptomycin administered to mice greatly reduced the coliform organisms with a concomitant rise in *Bacillus megatherium* which has been reported by McGinnis and co-workers(15) to produce large quantities of vitamin B_{12}. 4. Aureomycin may have a specific hematopoietic action not dependent upon its antibiotic activity. This seems unlikely, especially in view of the lack of response to aureomycin given intravenously in our last case. All other agents effective in the treatment of pernicious anemia have been much more effective when given parenterally than when given orally.

Conclusions. Four patients with Addisonian pernicious anemia in relapse and one patient with nutritional macrocytic anemia were treated with aureomycin given orally, with definite although submaximal hematological improvement. A fifth patient with Addisonian pernicious anemia was treated with aureomycin given intravenously with no response.

We are indebted to Mrs. Helen Jakubowski for performing the blood examinations.

12. Davis, B. D., and Mingioli, E. S., *J. Bact.*, in press.

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15. McGinnis, James, Stephenson, E. L., Levadie, B. T., Carver, J. S., Garibaldi, J. A., Ijichi, K., Snell, W. S., and Lewis, J. C., *Abstracts Am. Chem. Soc. Meeting*, Sept., 1949, p. 42.

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